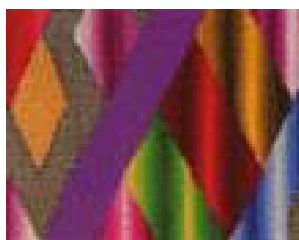




UNIVERSIDADE DO MINHO

ESCOLA DE CIÊNCIAS

ESCOLA DE ENGENHARIA



## Degradation of dyes with microorganisms

### Studies with ascomycete yeasts

Patrícia Alexandra da Costa Ferreira Ramalho

Braga, 2005

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Degradation of dyes with microorganisms  
Studies with ascomycete yeasts

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the PhD degree in Sciences

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## ABSTRACT

The capabilities of several yeasts were explored for the degradation of textile azo dyes. Through the initial work with *Candida zeylanoides* it has been possible to improve the composition of the culture medium to achieve the decolourisation of several model azo dyes. Total decolourisation times observed in culture media supplemented with 0.2mM dye ranged from 40 to 60 h. The initial decolourisation rates were  $14\text{--}52\mu\text{mol}(\text{g dry cell})^{-1}\cdot\text{h}^{-1}$ , depending on dye structure. In the course of decolourisation either metanilic or sulfanilic acids were detected in the supernatant fluid, showing that decolorization by this yeast strain was due to azo bond reduction. The azoreductase activity of the yeast cells was not significantly affected by pre-adaptation of the microorganism to the dyes.

The exploration of the species *Issatchenkia occidentalis* demonstrated that the extent of decolourisation of the dyes previously tested, in liquid media containing 0.2 mM dye and 2% glucose, reached more than 80% in a period of 15 h, essentially under microaerophilic conditions. Under anoxic conditions, decolourisation did not occur, even in the presence of pre-grown cells. Kinetic assays of azo reduction activities with *quasi*-resting cells demonstrated the following: (i) while the optimum pH depended on dye structure, the optimum pH range was observed in the acidic range; (ii) the maximum decolorizing activity occurred in the late exponential growth phase; and (iii) the temperature profile approaches the typical bell-shaped curve. These results indirectly suggested the involvement of an enzyme activity in azo dye reduction. The decolourising activity of *I. occidentalis* was still observed when the cells switched to aerobic respiration at the expense of ethanol, after glucose exhaustion in the culture medium, but ceased when all the ethanol was consumed, connecting the dye reduction to growth.

The efficiencies of *I. occidentalis* in the decolourisation of several azo dyes measured as the time required for colour loss  $\geq 98\%$  was shown to be linearly related to the reduction peak potential of the dyes. This contribution is a part of a paper which also examined the oxidative decolourisations by *Trametes villosa* laccase.

It was demonstrated that in *Saccharomyces cerevisiae* the ferric reductase system participates in the extracellular reduction of azo dyes. The *S. cerevisiae* mutant strains  $\Delta\text{fre1}$  and  $\Delta\text{fre1}\Delta\text{fre2}$ , but not  $\Delta\text{fre2}$ , showed a much reduced decolourising capability, suggesting that, under the conditions tested, Fre1p is a major component of the azo reductase activity. *FRE1* gene complemented the phenotype of *S. cerevisiae*  $\Delta\text{fre1}$  cells recovering the ability to grow in medium without externally added iron and to decolourise the dye, following a pattern similar to the one observed in the wild-type strain.

The decolourising capabilities of *C. zeylanoides*, *I. occidentalis* and *S. cerevisiae* CEN.PK 113-7D towards several azo dyes were compared. The presence of dyes and degradation products in the growth medium did not affect nor growth nor viability of cells. The specific degradation rates obtained showed that *S. cerevisiae* is much more efficient in the decolourisation of dyes I, II and amaranth. The relationship of  $\ln(\text{apparent first order decolourisation rate/specific growth rate})$  vs. the reduction peak potential was represented by a second order polynomial equation. This type of relation suggests that the decolourisation kinetics is energy-dependent. Assimilation experiments of the reduction products showed that all strains are able to use the formed amines as carbon and nitrogen sources. This represents the possibility of complete mineralization of the tested azo dyes. The use of alternative carbon sources was also explored. All the three strains presented the same behaviour. Besides glucose ethanol was the only substrate that allowed both growth and decolourisation.

A continuous process was developed to degrade azo dyes with a bacterial consortium isolated from a textile wastewater operating at pH 9 and 55°C with the objective of comparing it to a similar system with yeasts. The effects of hydraulic retention time (HRT), pH, temperature and presence of salts were studied. For the optimal operational conditions (pH 9 and HRT of 24 h) the efficiencies achieved were  $91.5 \pm 1.3\%$  for colour removal and  $89.0 \pm 0.4\%$  for COD removal. The system tolerated, with no significant decrease in colour removal efficiency 3% of  $\text{Na}_2\text{SO}_4$ ,  $\text{Na}_2\text{CO}_3$  or  $\text{NaCl}$ . The later salts, however, produced a reduction in COD removal of 30 and 50% respectively. The total suspended solids content in the outlet of the reactor changed widely during the operation with a mean value of  $0.54 \pm 0.22 \text{ g.L}^{-1}$ . The system proved to be very effective in the decolourisation of C.I. Reactive Black 5 (RB5) under alkaline conditions and high temperatures. The similar system with yeasts due to lack of time was not operated.

## RESUMO

O principal objectivo deste trabalho consistiu em explorar a capacidade de degradação de corantes azo têxteis de algumas leveduras. O trabalho inicial com uma estirpe da espécie *Candida zeylanoides* permitiu otimizar a composição do meio de cultura para conseguir a descoloração de alguns corantes azo modelo. Os tempos totais de descoloração, observados no meio de cultura suplementado com 0.2mM do corante, situaram-se entre as 40 e as 60h. As taxas iniciais de degradação foram 14–52  $\mu\text{mol} \cdot (\text{g peso seco})^{-1} \cdot \text{h}^{-1}$ , dependendo do corante usado. No decorrer do processo de descoloração detectou-se a presença dos ácidos metanílico e sulfanílico nos sobrenadantes dos meios, provando que a descoloração se deve à redução da ligação azo. A actividade de azo reductase não é significativamente afectada pela exposição prévia das células ao corante.

Os estudos levados a cabo com a levedura *Issatchenkia occidentalis* demonstraram que a extensão da descoloração dos corantes previamente testados, em meio líquido contendo 0.2mM de corante e 2% de glucose, atingiu mais de 80% num período de 15 horas, essencialmente em condições microaerofílicas. Sob condições anóxicas a descoloração não ocorreu, mesmo na presença de células pré-crescidas. Ensaio cinéticos da actividade de azo reductase demonstraram que: (i) o pH óptimo depende da estrutura do corante e situa-se na gama ácida; (ii) a actividade máxima de descoloração ocorre durante a fase exponencial tardia; e (iii) a actividade varia com a temperatura aproximadamente segundo uma curva em forma de sino. Estes resultados sugerem indirectamente o envolvimento de uma actividade enzimática na redução dos corantes azo. A actividade de descoloração de *I. occidentalis* manteve-se mesmo após a mudança para o metabolismo respiratório do etanol, após o esgotamento da glucose do meio, mas cessou quando todo o etanol foi consumido, ligando a redução dos corantes ao crescimento.

As eficiências de *I. occidentalis* na descoloração de vários corantes azo, medidas pelo tempo necessário para atingir uma descoloração  $\geq 98\%$ , estão linearmente relacionadas com o potencial do pico de redução do corante. Esta contribuição é parte integrante de um artigo que também examina a descoloração oxidativa pela laccase de *Trametes villosa*.

Em *Saccharomyces cerevisiae* o sistema da ferri-reductase participa na redução extracelular de corantes azo. Os mutantes  $\Delta fre1$  e  $\Delta fre1\Delta fre2$ , mas não o  $\Delta fre2$ , da estirpe de *S. cerevisiae* utilizada mostraram uma capacidade muito reduzida de descoloração, sugerindo que, nas condições testadas, Fre1p é o maior componente da actividade de azo reductase. O gene *FRE1* complementou o fenotipo do mutante  $\Delta fre1$ , recuperando a capacidade de crescer em meio sem adição de ferro e de degradar o corante, seguindo um padrão semelhante ao observado na estirpe selvagem.



As estirpes de *C. zeylanoides*, *I. occidentalis* e *S. cerevisiae* acima referidas foram comparadas quanto à sua capacidade de descoloração de vários corantes. A presença dos corantes ou dos produtos da sua degradação no meio de cultura não afectou nem o crescimento nem a viabilidade das células. As taxas específicas de degradação obtidas mostraram que a estirpe de *S. cerevisiae* testada é mais eficiente na descoloração dos corantes I, II e Amarante. A relação entre  $\ln(\text{constante de descoloração de 1}^{\text{a}} \text{ ordem aparente/taxa específica de crescimento})$  vs. potencial de pico de redução do corante é representada por uma equação polinomial de segunda ordem. Este tipo de dependência sugere que o factor energético é determinante na cinética do processo de descoloração. Experiências de assimilação dos produtos de redução demonstraram que todas as estirpes utilizam as aminas formadas como fonte de carbono e azoto. Isto representa a possibilidade de mineralização completa dos corantes. A utilização de fontes de carbono alternativas também foi explorada. Todas as três estirpes utilizadas apresentaram um comportamento semelhante. Para além da glucose, o etanol foi o único substrato que permitiu o crescimento e a descoloração.

Foi desenvolvido um processo contínuo para degradar corantes azo com um consórcio de bactérias isolado a partir de um efluente têxtil para operar a pH 9 e 55°C, tendo sido estudados os efeitos do tempo de retenção hidráulico ( $\theta$ ), pH, temperatura e presença de sais. Para as condições óptimas de operação (pH 9 e  $\theta=24\text{h}$ ) as eficiências alcançadas foram  $91.5\pm1.3\%$  para a remoção da cor e  $89.0\pm0.4\%$  para a remoção da carência química de oxigénio (CQO). O sistema tolerou, sem significativa redução da eficiência de remoção de cor, 3% de  $\text{Na}_2\text{SO}_4$ ,  $\text{Na}_2\text{CO}_3$  ou  $\text{NaCl}$ . Os últimos sais provocaram no entanto uma redução da eficiência de remoção de CQO de 30 e 50% respectivamente. O conteúdo em sólidos suspensos totais à saída do reactor variou bastante durante toda a operação do mesmo, tendo apresentado um valor médio de  $0.54\pm0.22 \text{ g.L}^{-1}$ . O sistema provou ser eficiente para a descoloração do corante C.I. Reactive Black 5 (RB5) em condições alcalinas e a temperaturas elevadas. Os dados obtidos neste estudo servirão futuramente para comparação com um sistema semelhante utilizando leveduras.

## PUBLICATIONS

### Papers published in peer-reviewed journals

- Ramalho, P. A., Scholze, H., Cardoso, M. H., Ramalho, M. T. & Oliveira-Campos, A. M. (2002). Improved conditions for the aerobic reductive decolourisation of azo dyes by *Candida zeylanoides*. *Enzyme and Microbial Technology* 31, 848-854.
- Ramalho, P. A., Cardoso, M. H., Cavaco-Paulo, A. & Ramalho, M. T. (2004). Characterization of azo reduction activity in a novel ascomycete yeast strain. *Appl Environ Microbiol* 70, 2279-88.
- Zille, A., Ramalho, P., Tzanov, T., Millward, R., Aires, V., Cardoso, M. H., Ramalho, M. T., Gubitz, G. M. & Cavaco-Paulo, A. (2004). Predicting dye biodegradation from redox potentials. *Biotechnol Prog* 20, 1588-92.
- Ramalho, P. A., Paiva, S., Cavaco-Paulo, A., Casal, M., Cardoso, M. H. & Ramalho, M. T. (2005). Azo reductase activity of intact *Saccharomyces cerevisiae* cells is dependent on the Fre1p component of the plasma membrane ferric reductase (accepted for publication in Applied and Environmental Microbiology).

### Papers to be published in peer-reviewed journals

- Ramalho, P. A., Cardoso, M. H., Ramalho, M. T., Gubitz, G. M. & Cavaco-Paulo, A. Decolourisation of reactive azo dyes in alkaline textile effluents with a bacterial consortium (submitted to Enzyme and Microbial Technology EMT/patramalho.AT.bio.uminho.pt/20041006).
- Ramalho, P. A., Cavaco-Paulo, A., Ramalho, M. T. & Cardoso, M. H. Comparison of the azoreductase activities and assimilation of dye reduction products by three ascomycete yeast strains (in preparation).

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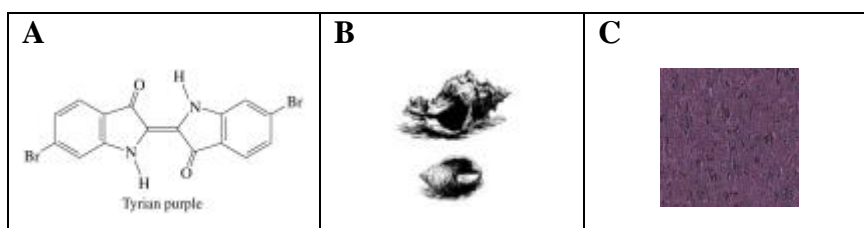
# **CHAPTER 1**

## **INTRODUCTION**

# 1. INTRODUCTION

## 1.1 HISTORY OF DYES

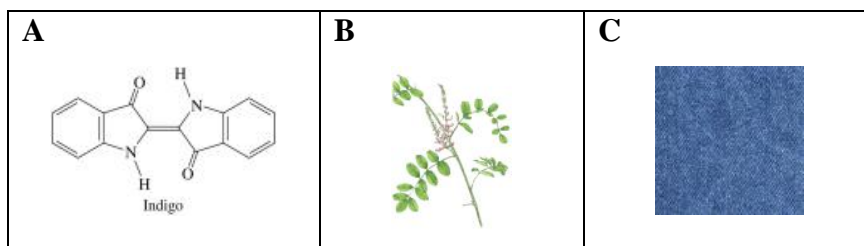
Ever since the beginning of mankind, people have been using colorants for painting and dyeing their surroundings, their skins and their clothes. The first evidence of the use of colorant materials by man goes as far as 15000-9000 BC, in the walls of the Altamira cave in Spain. The drawings were performed with inorganic pigments like soot, manganese oxide, hematite and ochre. Historically there is a dye, derived from animal sources (molluscs), that is very important, although presently has no relevance and it's not commercially available. It is Tyrian Purple (see Figure 1) and the pigment itself is not in the mollusc; however, when the precursor is extracted it can be converted to the dye by air or light. The presence of this dye goes as far as 1400 BC in the Late Bronze Age as found recently in Lebanon. It has always been rare and costly being used by Roman emperors and high ranking ecclesiastics (Clark *et al.* 1993).



**Figure 1:** A - Chemical structure of tyrian purple; B - Sea shells from which tyrian purple was extracted; C - A purple-dyed fabric.

Another ancient dye that is still in use, although not from natural origin nowadays, is indigo. It was extracted from *Indigofera tinctoria* by fermentation and had a characteristic blue colour (see Figure 2). It was used as a pigment by the Romans because it had to be chemically reduced to become water soluble. It was firstly synthetically produced by Adolf von Baeyer in 1880, and actually is used to dye denim (Clark *et al.* 1993).

Up to the end of the nineteenth century natural dyes, obtained mainly from plants (roots, stems, leaves, flowers, fruits, seeds and lichens – Ingamells 1993), were the main colorants available for textile dyeing procedures. The main disadvantages of the use of natural dyes are the need for several steps in the dyeing process, the diversity of sources and related application procedures, the rapid change in trends and the demand for good fastness properties on different substrates that would require a complete database describing possible applications (Bechtold *et al.* 2003).



**Figure 2:** A - Chemical structure of indigo; B – *Indigofera tinctorium*; C - Denim.

The pioneering synthesis of mauveine by W. H. Perkins started the era of synthetic dyes, with chemical and physical properties better suited to contemporary demands, better level of quality and more reproducible techniques of application. It also allowed the development and extension of the use of particular products. For example, the development of synthetic fibres such as polyester and cellulose triacetate would have been severely hindered without the design and synthesis of dyes with appropriate properties (Ingamells 1993).

Since then thousands of dyes have been synthesised, and dye manufacture has become a significant part of the chemical industry. Nowadays, when care of the environment is a major issue, it is tempting to assume that the use of natural colours is an environmental friendly alternative to present-day practice. There are several groups studying the use of natural dyes in modern dyeing industry (Tsatsaroni and Liakopoulos-Kyriakides 1995, Angelini *et al.* 1997, Ishigami and Suzuki 1997, Angelini *et al.* 2003, Bermejo *et al.* 2003, Kim *et al.* 2004b, Paul *et al.* 2004, Kamel *et al.* 2005, Singh *et al.* 2005). Some of the advantages of the use of this type of compounds are the absence of toxicity upon humans, the use of sustainable sources and the fit into the natural pathways of biodegradation of the released dyebaths.

## 1.2 STRUCTURES AND USES OF DYES

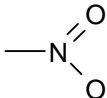
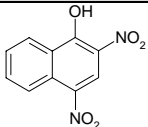
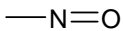
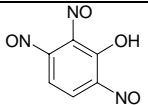
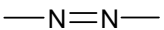
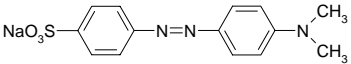
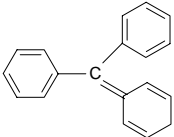
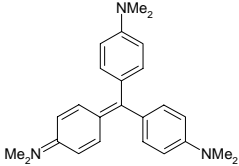
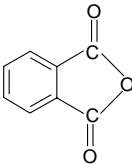
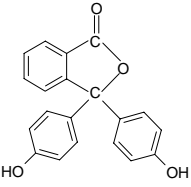
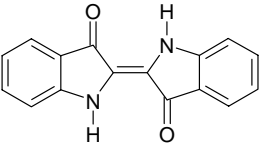
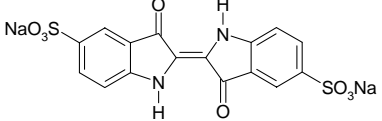
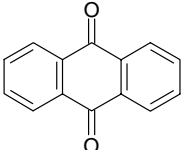
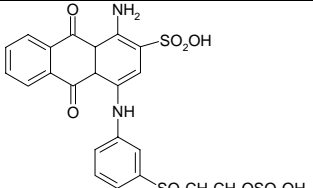
Dyes are compounds that absorb light with wavelengths in the visible range, i.e., 400 to 700 nm (de las Marias 1976, van der Zee 2002). The major structure element responsible for light absorption in dye molecules is the chromophore group, i.e. a delocalised electron system with conjugated double bonds. The absorption of UV/Vis radiation by an organic molecule is associated with electronic transitions between molecular orbitals. The energy of the absorbed radiation is given by:

$$\Delta E = E_1 - E_0 = h\nu = hc / \lambda$$

where  $E_0$  is the energy corresponding to the fundamental state of the molecule (J),  $E_1$  is the excited state energy (J),  $h$  is the Planck's constant ( $6.626 \times 10^{-34}$  Js),  $\nu$  is the electromagnetic

radiation frequency (Hz),  $c$  is the light velocity ( $3 \times 10^8 \text{ m.s}^{-1}$ ) and  $\lambda$  is the wavelength (nm). The more extended the electronic delocalisation, the lower is the transition energy and the higher is the wavelength. To allow delocalization of the electrons double bonds must alternate with single bonds. In the case of synthetic dyes, delocalization is also promoted by benzene or naphthalene rings (Rocha Gomes 2001). Chromophores frequently contain heteroatoms as N, O and S, with non-bonding electrons. By incorporating these electrons into the delocalised system in the aryl rings, the energy of the electron cloud is modified, the wavelength of the absorbed radiation will shift towards the visible range, and the compound will be coloured. In many cases dyes contain

**Table 1:** Classification and examples of dyes according to the chromophore present

CLASS	CHROMOPHORE	EXAMPLE
Nitro dyes		 C.I. Acid Yellow 24
Nitroso dyes		 Fast Green O
Azo dyes		 Methyl Orange
Triphenyl methyl dyes		 C.I. Basic Violet 3
Phthalein dyes		 Phenolphthalein
Indigoid dyes		 C.I. Acid Blue 71
Anthraquinone dyes		 C.I. Reactive Blue 19

additional groups called auxochromes, which are electron withdrawing or electron donating substituents that cause or intensify the colour of the chromophore by altering the overall energy of the electron system. The most important auxochrome groups are: hydroxyl and derivatives, -OH, -OR; amino and derivatives, -NH<sub>2</sub>, -NHR, -NHR<sub>2</sub>; sulphonic, -SO<sub>3</sub>H; carboxylic, -COOH; and sulphide, -SR (de las Marias 1976, van der Zee 2002). Some auxochromes also increase the dye affinity for the fibre (natural or synthetic). Natural fibres are based on cellulose (polymeric linear chains of glucose) – cotton and linen - or proteins – wool and silk. Synthetic fibres are for instance viscose, cellulose acetate, polyamide, polyester and acrylic (Guaratini and Zanoni 2000). Common classes of dyes, based on the chromophore present, are shown in Table 1.

According to the Colour Index dyes can be classified on the basis of colour and application method. Various attractive forces have the potential of binding dyes to fibres, and often more than one type of chemical bonding can operate with the same dye-fibre combination. The dominant force depends on the chemical character of the fibre and the chemical groups in the dye molecule. The types of bonds established between the dye and the fibre, by increasing relative strength of the bond, can be: Van der Waals, hydrogen, ionic or covalent (Ingamels 1993, Guaratini and Zanoni 2000, Rocha Gomes 2001). According to the application categories dyes can be classified as seen in Table 2.

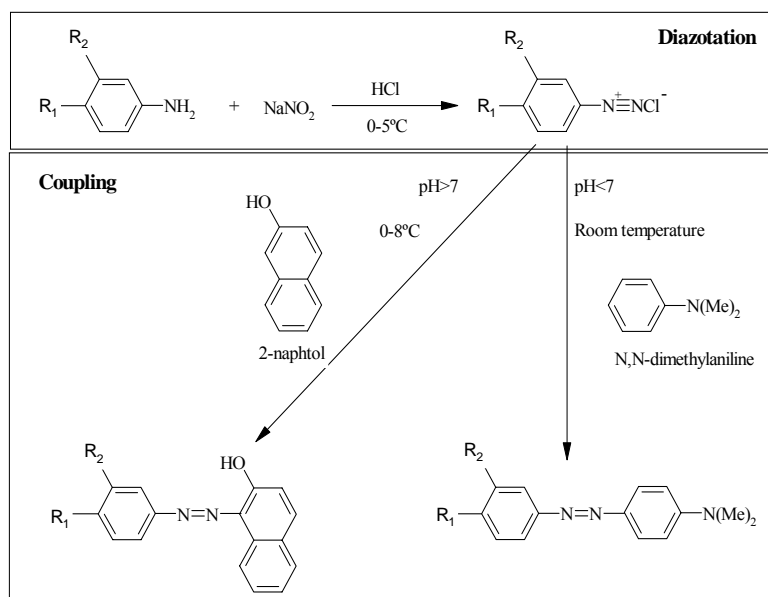
Dyes are used in textile industry, leather tanning industry, paper production, food technology, agricultural research, light-harvesting arrays, photoelectrochemical cells, hair colouring and cosmetics. Moreover these compounds have been employed for the control of the efficacy of sewage and wastewater treatment, for the determination of specific surface area of activated sludge and for ground water tracing (Forgacs *et al.* 2004). Due to the large amounts used, the most significant industrial use is in textile dyeing.

Azo dyes are the most widely used among synthetic dyes, representing almost 70% of the textile dyestuffs produced (Knackmuss 1996). They are easy to synthesize, have low cost, are stable, can be used to colour several materials (textile, leather, plastic, food) and allow a great variety of colours and shades. They have in their molecule one or more azo groups. They are obtained from the coupling of diazonium salts with aromatic amines, phenols, naphthols or aliphatic enols. Coupling usually takes place in the *para* position in respect to the amino or hydroxyl group or in the *orto* position if the latter is occupied. The diazonium salts are obtained from the reaction of sodium nitrite with an amine solution with a mineral acid, preferably HCl (de las Marias 1976, Zollinger *et al.* 1991) – Figure 3. The structural class of azo dyes includes dyes from different application classes, namely, acid, basic, metal complex, reactive and mordant (de las Marias 1976).



**Table 2:** Application categories of dyes (adapted from O'Neill *et al.* 1999 and Rocha Gomes 2001)

TYPE OF DYE	CHARACTERISTICS	SUBSTRATES
Acid	When in solution are negatively charged; bind to the cationic $\text{NH}_3^+$ -groups present in fibres	Nylon, wool, polyamide, silk, modified acryl, paper, inks and leather
Reactive	Form covalent bonds with OH-, NH- or SH- groups	Cotton, wool, silk and nylon
Metal complex	Strong complexes of one metal ion (usually chromium, copper, cobalt or nickel) and one or two dye molecules (acid or reactive)	Silk, wool and polyamide
Direct	Large molecules bound by Van der Waals forces to the fibre	Cellulose fibres, cotton, viscose, paper, leather and nylon
Basic	Cationic compounds that bind to the acid groups of the fibre	Synthetic fibres, paper and inks
Mordant	Require the addition of a chemical that combines with the dye and the fibre, like tannic acid, alum, chrome alum, and other salts of aluminium, chromium, copper, iron, potassium, and tin	Wool, leather, silk, paper, modified cellulose fibres and anodised aluminium
Disperse	Scarcely soluble dyes that penetrate the fibre through fibres swelling	Polyester, polyamide, acetate, acrylic and plastics
Pigment	Insoluble, non-ionic compounds or insoluble salts that retain their crystalline or particulate structure throughout their application	Paints, inks, plastics and textiles
Vat	Insoluble coloured dyes which on reduction give soluble colourless forms (leuco form) with affinity for the fibre; on exposure to air are reoxidised	Cellulose fibres, cotton, viscose and wool
Azoic and Ingrain	Insoluble products of a reaction between a coupling component and a diazotised aromatic amine that occurs in the fibre	Cotton, viscose, cellulose acetate and polyester
Sulphur	Complex polymeric aromatics with heterocyclic S-containing rings	Cellulose fibres, cotton and viscose
Solvent	Non ionic dyes that dissolve the substrate to which they bind	Plastics, gasoline, varnish, lacquer, stains, inks, oils, waxes and fats
Fluorescence brighteners	Mask the yellowish tint of natural fibres	Soaps and detergents, all fibres, oils, paints and plastics
Food	Non-toxic and not used as textile dyes	Food
Natural	Obtained mainly from plants	Food, cotton, wool, silk, polyester, polyamide and polyacrylonitrile



**Figure 3:** Synthesis of azo dyes.

### 1.3 ECOLOGICAL ASPECTS

The most problematic industries in terms of dye release to the environment in the form of wastewater are the production of dyes and the dyeing industry. The uncontrolled release of these compounds in the environment causes severe problems. Since they are designed to be chemically and photolytically stable they are highly persistent in natural environments. For instance, the half-life of hydrolised Reactive Blue 19 is about 46 years at pH 7 and 25°C (Hao *et al.* 2000). In fact they are xenobiotic compounds because they do not exist as natural products and therefore contain structural elements that cannot be synthesized biochemically (Stolz 2001, Rieger *et al.* 2002). During evolution of catabolic enzymes and pathways microorganisms were not exposed to these structures and have not developed the capability to use those compounds as sole sources of carbon and energy.

Dyes, by decreasing light absorption, may significantly affect photosynthetic activity of aquatic life and may be toxic due to the presence of aromatics or heavy metals (Banat *et al.* 1996, Slokar and Le Marechal 1998, Zanoni and Carneiro 2001, Kunz *et al.* 2002, Carneiro *et al.* 2004). The possibility of bioaccumulation in fish was considered but the few studies on the subject did not confirm this hypothesis (Anliker and Moser 1987, Anliker *et al.* 1988, Law 1995, van der Zee 2002).

In the case of azo dyes, their degradation under anaerobic conditions in sediments causes the release of potentially carcinogenic and mutagenic aromatic amines, whose carcinogenicity and mutagenicity is further discussed in the next section.

## 1.4 TOXICOLOGICAL ASPECTS

The acute toxicity of azo dyes, as defined by the EU criteria for the classification of dangerous substances, is rather low (Øllgaard *et al.* 1998, van der Zee 2002). Only a few azo dyes showed LD50 (lethal dose that kills half of the tested population) values below 250 mg / kg body weight (*in van der Zee* 2002). However, occupational sensitisation to azo dyes has been seen in the textile industry since 1930 (Foussereau *et al.* 1982). Especially some disperse dyes with monoazo or anthraquinone structures have been found to cause allergic reactions, i.e. eczema or contact dermatitis (Hatch and Maibach 1995). Nevertheless attributing an allergy to a particular azo dye is a complex and difficult process due to the large number of azo dyes that exist (*c.a.* 2000), the different names that different fabricants attribute to the same azo dye and impurities that usually azo dyes carry along.

Following oral exposure azo dyes are metabolised to aromatic amines by intestinal microflora or liver azoreductases. The soluble aminosulphonates are usually quickly excreted, whereas those derived from aniline, toluene, benzidine and naphthalene have been shown to have carcinogenic properties. However these properties are attributed to further metabolism (oxidation to N-hydroxy-compounds) by mammalian cytochrome P-450 enzymes. For instance, methemoglobinemia results from the oxidation of iron(II) to iron(III) in haemoglobin, which prevents oxygen binding (Lin and Wu 1973).

The biologically active dyes were mainly limited to those compounds containing *p*-phenylenediamine and benzidine moieties. It was found that for the phenylenediamine component methylation or substitution of a nitro group for an amino group did not decrease mutagenicity. However sulphonation, carboxylation or diamination lead to a decrease in the mutagenicity (Chung and Cerniglia 1992).

## 1.5 CHARACTERISATION OF TEXTILE WASTEWATERS

Wet processing in textile industry generates large amounts of a wastewater whose pollution load arises not only from the removal of impurities from the raw materials themselves but also from the residual chemical reagents used for processing (Table 3). The extreme diversity of raw materials and production schemes employed poses problems in assessing effluent characteristics and subsequently defining pollution control technologies (Correia *et al.* 1994). Colour is one of the major problems of these types of wastewaters. During textile processing, inefficiencies in dyeing result in large amounts of dyestuff being directly lost to the wastewater, which

ultimately finds its way into the environment. The amount of dye lost is dependent upon the dye application class, varying from only 2% loss when using basic dyes to a 50% loss when certain reactive dyes are used (O'Neill *et al.* 1999, McMullan *et al.* 2001, Pearce *et al.* 2003).

**Table 3:** Major pollutant types in textile wastewater, chemical types and process of origin (adapted from Delée *et al.* 1998).

POLLUTANTS	CHEMICAL TYPES	PROCESS OF ORIGIN
Organic load	Starches, enzymes, fats, greases, waxes, surfactants and acetic acid	Ds, S, W, Dy
Colour	Dyes, scoured wool impurities	Dy, S
Nutrients (N, P)	Ammonium salts, urea, phosphate-based buffers and sequestrants	Dy
pH and salts	NaOH, mineral/organic acids, sodium chloride, silicate, sulphate, carbonate	S, Ds, B, M, Dy, N
Sulphur	Sulphate, sulphite and hydrosulphite salts, sulphuric acid	Dy
Toxic compounds	Heavy metals, reducing agents (sulphide), oxidising agents (chlorite, peroxide, dichromate, persulphate), biocides, quaternary ammonium salts	Ds, B, Dy, F
Refractory organics	Surfactants, dyes, resins, synthetic sizes (PVA), chlorinated organic compounds, carrier organic solvents	S, Ds, B, Dy, W, F

**Ds** – desizing; **S** – scouring; **W** – washing; **Dy** – dyeing; **B** – bleaching; **M** – mercerising; **N** – neutralisation; **F** - finishing

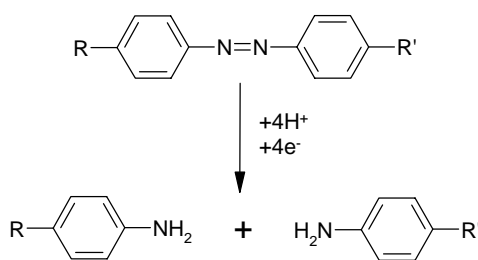
## 1.6 TREATMENT OF TEXTILE WASTEWATERS

Colour is the first contaminant to be recognized in wastewater and has to be removed before discharging into water bodies. The presence of very small amounts of dyes in water (less than 1 ppm for some dyes) is highly visible and affects the aesthetic quality water transparency and gas solubility in lakes, rivers and other water bodies. The removal of colour is often more important than the removal of the soluble colourless organic substances, which usually contribute the major fraction of the biochemical oxygen demand (BOD). Methods for the removal of BOD from most effluents are fairly well established. Dyes, however, are more difficult to treat because of their synthetic origin and complex aromatic molecular structures (Banat *et al.* 1996).

The electron-withdrawal character of the azo-group generates electron deficiency. This makes the compounds less susceptible to oxidative catabolism and as a consequence many of these chemicals tend to persist under aerobic environmental conditions (Knackmuss 1996). Dyes must have a high degree of chemical and photolytic stability in order to be useful, due to the

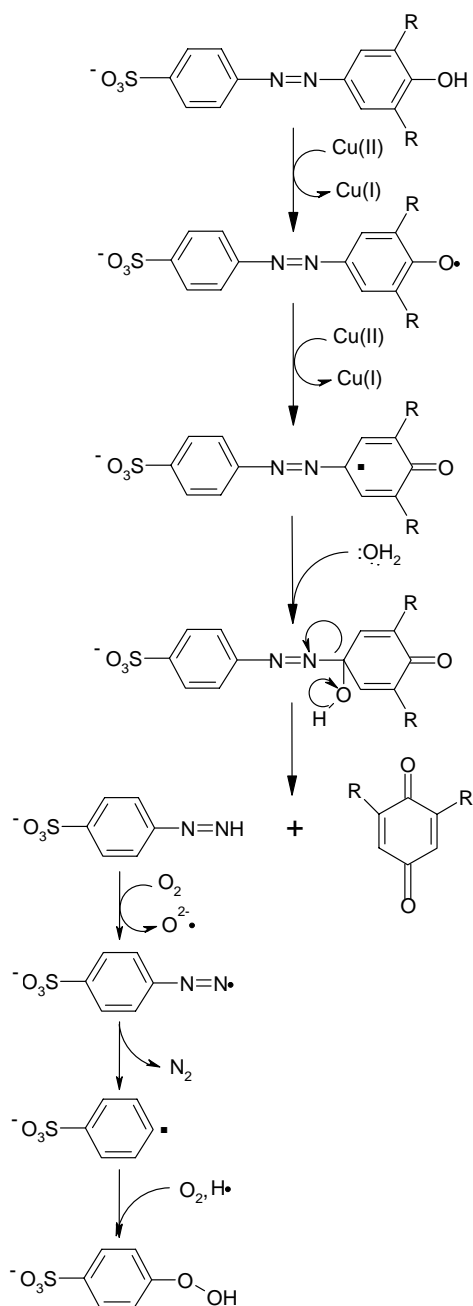
harshness of the conditions to which they are submitted during and after the dyeing process (light, bases, peroxides, and others (Rocha Gomes 2001)). Therefore they will, in general, give negative results in short-term tests for aerobic biodegradability (*in* Øllgaard et al. 1998). Stability against microbial attack is also a required feature of azo dyes (Pagga and Brown 1986), because it will prolong the lifetime of the products in which azo dyes are applied.

When designing the solution for a particular wastewater there are several conditionings that should be considered in this choice. Due to the high variability of composition of this type of wastewater, not only in quantity but mainly in quality, usually there is the need of more than one type of treatment to achieve the necessary quality of treatment. There are two possible locations for any technology that could be used to remove the colour that is present in the wastewater. The first possibility is in the dyehouse, allowing the partial or full re-use of water. This possibility deals with a considerable smaller amount of wastewater than the next possibility which is the treatment on a municipal (or particular) wastewater treatment plant, usually with a biological step. In this case, the coloured wastewater is mixed with domestic wastewater to provide organic load for the biological treatment. And in this hypothesis it can be used before the current biological or chemical treatment or as a final polishing step (Pearce *et al.* 2003). A wide range of methods has been developed for the removal of synthetic dyes from waters and wastewaters to decrease their impact on the environment. They are divided in three major categories: physical, chemical or biological. They are described and discussed in detail in the next sections. Biological and chemical methods involve the destruction of the dye molecule, whilst physical methods usually transfer the pollutant to another phase. Destruction of azo dyes can be accomplished by reduction or by oxidation. The reduction of azo dyes generates aromatic amines (see Figure 4).



**Figure 4:** Reduction of an azo dye.

The oxidative mechanisms are difficult to establish due to the high reactivity of the free radicals usually involved in the process. The oxidation of phenolic azo dyes by laccase was described by the following mechanism:



**Figure 5:** Oxidation of a phenolic azo dye by laccase (adapted from Chivukula and Renganathan 1995).

### 1.6.1 PHYSICAL TREATMENTS

**Sorption.** Adsorption techniques have recently gained a considerable importance due to their efficiency in the removal of pollutants too stable for conventional methods (Robinson *et al.* 2001, Aksu 2005). Remediation based on sorption phenomena involves binding of soluble or suspended pollutants to a solid organic or inorganic matrix. Binding to the matrix, depending on its

composition and on pollutant structure, may be due to several types of interactions, e.g. electrostatic, ionic exchange, van der Waals, complexation or chelation. Decolourisation of wastewater by this process is influenced by many factors such as sorbent surface area, particle size, contact time, temperature, pH and presence of salts, surfactants and metals (*in* Robinson *et al.* 2001). It must be emphasized, however, that sorption processes merely transfers pollutants from one phase to another and therefore invariably generate sludge that must be disposed off, or regenerated, by some other process. Most adsorbents are not equally effective towards different types of dyes (van der Zee 2002). The efficiency of one sorbent it is frequently assessed using the Freundlich empirical equation:

$$X/M = KC^{1/n}$$

where:

X = amount of impurity adsorbed

M = mass of adsorbent

C = equilibrium concentration of impurity in solution

K, n = constants

From a logarithmic plot of data it is possible to determine the adsorbent capacity of the material at specified equilibrium concentrations of particular impurities (Reife and Freeman *in* Reife and Freeman 1996).

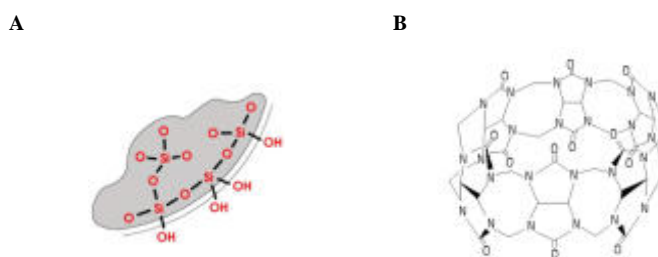
Adsorption methods have some drawbacks. Since adsorption processes are not selective, the other components of the wastewater can compete for the adsorbing sites reducing the dye binding capacity of the adsorbent. Moreover, an adsorption process removes the synthetic dyes from the wastewater by concentrating them on the surface retaining their structure practically unchanged. When the support is to be regenerated, the fate of the resulting concentrated sludge of dyes presents a problem of correct disposal (Forgacs *et al.* 2004).

Sorbents can be inorganic or organic materials, or biomass (living or dead). Inorganic sorbents have the advantage of good mechanical and chemical stability, high specific surface area and resistance to microbiological degradation (Forgacs *et al.* 2004).

Activated carbon is a versatile adsorbent because its superficial chemical groups can be modified according to the physical-chemical treatment to which it is submitted, e.g. HNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>, NH<sub>3</sub> or thermal treatments under H<sub>2</sub> or N<sub>2</sub> (Pereira *et al.* 2003, Faria *et al.* 2004). It is one of the most widely used adsorbent for the removal of different classes of dyes but, because it is expensive and the cost of its regeneration is also high, it is usually applied as a tertiary/polishing treatment (Golob and Ojstršek 2005). Recent works describe attempts to produce activated carbons from cheap and readily available sources, like sawdust and rice-husk (Malik 2003, Malik 2004). It is

highly effective for adsorbing cationic, mordant and acid dyes and, to slightly lesser extent, disperse, direct, vat, reactive and pigment dyes (*in Robinson et al. 2001*).

In the last years there has been an effort towards the application of cheaper adsorbing materials for colour removal in wastewaters. There are studies describing the adsorbing capacities of peat (Ramakrishna and Viraraghavan 1997, Ho and McKay 1998, Ho and McKay 2003, Sun and Yang 2003) and of several agricultural by-products, as wood chips (Nigam *et al.* 2000), corn cobs (Nigam *et al.* 2000, Robinson *et al.* 2002), rice hulls (Nawar and Doma 1989), peanut hulls (Gong *et al.* 2005), and also aquatic plants (Waranusantigul *et al.* 2003). These materials are so cheap that regeneration is not necessary and the potential exists for dye-adsorbed materials to be used as substrates in solid-state fermentations for protein enrichment (Robinson *et al.* 2001). There are also cheap materials that can be used like modified starch (Delval *et al.* 2002, Riauka *et al.* 2004), fly ash and coal (Gupta *et al.* 1990, Woolard *et al.* 2002, Janos *et al.* 2003), clay (Shawabkeh and Tutunji 2003, Alkan *et al.* 2004, Wibulswas 2004) and silica gel (Ueno *et al.* 1986, Parida and Mishra 1996, Wu *et al.* 2004) – Figure 6.A.



**Figure 6.** Structures of silica (A) and cucurbituril (B).

Cucurbituril, a cyclic polymer of glycoluril and formaldehyde (Karcher *et al.* 1999) with a hydrophobic cavity and low solubility in aqueous solutions (Slokar and Le Marechal 1998) – see Figure 6.B - has also been used for colour removal. It forms insoluble complexes with the dye molecules and can be used as an aqueous solution or in the solid state. Almost complete decolourization can be achieved with all dye classes. Solid cucurbituril can be regenerated with ozone and peracetic acid (Slokar and Le Marechal 1998). One advantage of its use is that the presence of other organic substances in the water does not interfere with the formation of complexes, since it forms complexes with various organic compounds (Slokar and Le Marechal 1998, Karcher *et al.* 1999). Cost is its major disadvantage (Robinson *et al.* 2001).

The term biosorption refers to metabolism-independent processes taking place essentially in the cell wall of non-growing or non-living microbial mass. The main advantages of biosorption are high selectivity and efficiency, cost effectiveness and good removal performance; raw materials which are either abundant or wastes from industrial operations (fermentation wastes, activated sludge process wastes) can be used as biosorbents presenting performances often comparable



with those of ion exchange resins (Aksu 2005). Both living and death (heat killed, dried, acid and/or otherwise chemically treated) biomass can be used to remove hazardous organics and is effective when conditions are not favourable for the growth and maintenance of the microbial population (Robinson *et al.* 2001). **Bacteria** (Tai-Lee 1996, Walker and Weatherley 2000, Mahdavi Talarposhti *et al.* 2001), **fungi** (Mittal and Gupta 1996, Tatarko and Bumpus 1998, Fu and Viraraghavan 2001, Fu and Viraraghavan 2002, Zhang *et al.* 2003), **yeasts** (Bustard *et al.* 1998, Meehan *et al.* 2000, Donmez 2002, Aksu and Donmez 2003, Aksu and Donmez 2004) and **algae** (Aksu and Tezer 2005) have been described as good adsorbents, with biosorption capacities up to 640mg dye/g biomass (Aksu 2005). Nevertheless since textile dyes vary greatly in their chemistries, their interaction with the microorganisms will depend on the chemical structure of the dye, the specific chemistry of the microbial biomass and the characteristics of the dye solution or wastewater (Aksu 2005).

**Membrane filtration.** This technology has emerged as a feasible alternative to conventional treatment processes of dye wastewater and has proven to save operation costs and water consumptions by water recycling (Sójka-Ledakowicz *et al.* 1998, Koyunco *et al.* 2001, Kim *et al.* 2004a, Koyunco *et al.* 2004). Usually this technique is applied as a tertiary/final treatment after biological and/or

<i>Types of contaminants</i>				
<i>Molecular weight (Da)</i>	200	2000	20000	200000
<i>Pore size (nm)</i>	1	10	100	1000
<i>Separation process (applied pressures)</i>				

**Figure 7:** Filtration processes and characteristics (adapted from Madaeni 1999 and Elliott *in* Reife and Freeman 1996).

physical-chemical treatments (Ciardelli *et al.* 2000, Marcucci *et al.* 2001). It has also been used to concentrate and purify dyes in the manufacture of these compounds (Crossley 2002, Kim *et al.* 2004a, Koyuncu *et al.* 2004). These techniques allow, when not applied as end-of-pipe solutions, the recovery and reuse of some reagents (Sójka-Ledakowicz *et al.* 1998, Koyuncu *et al.* 2001, Marcucci *et al.* 2001, van der Zee 2002). The existing types of filtration are shown and characterized in Figure 7.

**Ion exchange.** Standard ion exchange systems have not been widely used for treatment of dye-containing effluents, mainly due to the opinion that ion exchangers cannot accommodate a wide range of dyes and dyeing conditions, and that their performance was greatly affected by the presence of additives in the wastewater (Slokar and Le Marechal 1998). In this technique wastewater is passed over the ion exchanger resin until all available exchange sites are saturated. Both anionic and cationic dyes are efficiently removed by this method. A disadvantage of this method is the high cost of organic solvents to regenerate the ion-exchanger (Slokar and Le Marechal 1998, Robinson *et al.* 2001).

**Coagulation/Floculation.** This method is often applied in the treatment of different types of wastewaters and it is used to enhance the degree of removal of total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and colour (Semerjian and Ayoub 2003, Allegre *et al.* 2004, Peres *et al.* 2004, Aguilar *et al.* 2005). The first step, coagulation, consists in the addition of a coagulant to the wastewater and mixing. This coagulant destabilizes the colloidal particles that exist in the suspension, allowing particle agglomeration. Flocculation is the physical process of bringing the destabilized particles in contact to form larger flocs that can be more easily removed from the solution (van der Zee 2002, Semerjian and Ayoub 2003, Golob and Ojstršek 2005). This is usually achieved by a slow mixing step. The most commonly used inorganic coagulants/flocculants are: trivalent salts of iron [ $\text{FeCl}_3$ ,  $\text{Fe}_2(\text{SO}_4)_3$ ] and aluminium [ $\text{Al}(\text{SO}_4)_3$ ]; ferrous sulphate  $\text{Fe}(\text{SO}_4)$ ; and calcium hydroxide/lime [ $\text{Ca}(\text{OH})_2$ ]. These are often used with various coagulant aids, such as synthetic polyelectrolytes (anionic, cationic or non-ionic polymers), fly ash and clay (Tchobanoglous and Burton 1991, van der Zee 2002, Semerjian and Ayoub 2003). Inorganic compounds are, however, generally not very suitable to remove highly soluble (sulphonated) dyes from solution unless large quantities are dosed (Hao *et al.* 2000). The major disadvantage of the use of this process is the amount of useless and even toxic sludge that needs to be correctly disposed (Motamedi 1975, Jeworski and Heinzle 2000, Chu 2001, Aguilar *et al.* 2002, van der Zee 2002, Papić *et al.* 2004) and the possibility of a secondary pollution problem (due to excess use of chemicals). Recently some organic polymers have been developed with good dye coagulant properties and a relatively low sludge production (van der Zee 2002).

### 1.6.2 CHEMICAL TREATMENTS

**Fenton's reagent.**  $\text{H}_2\text{O}_2$ -Fe(II) salts are used when the wastewater is resistant to biological treatment or is toxic to the biomass (Slokar and Le Marechal 1998). The reaction, that occurs at acidic pH, results in the formation of a strong oxidative hydroxyl radical ( $\text{HO}^\cdot$ ) and ferric iron (from the oxidation of ferrous iron). Both forms of iron are coagulants thus conferring the dual function of oxidation and coagulation to this process (Kang *et al.* 2002). Therefore the mechanism of colour removal involves also sorption or bonding of the dissolved dyes to flocs that are formed. The formation of large amounts of sludge concentrated in dyes and iron is the main disadvantage of this process, carrying disposal problems (Slokar and Le Marechal 1998, Robinson *et al.* 2001). As far as colour removal is concerned, the method is suitable for different dye classes. Reactive, direct, metal-complex, pigment, disperse and vat dyes have good decolouration rates (Slokar and Le Marechal 1998).

**Ozone.** This is a very good oxidizing agent due to its high instability (reduction potential 2.07V) when compared to chlorine (1.36V) and  $\text{H}_2\text{O}_2$  (1.78V). It degrades a high number of pollutants like phenols, pesticides and aromatic hydrocarbons and it is used since the early 1970s in wastewater treatment (Robinson *et al.* 2001, Özbelge *et al.* 2002, Pera-Titus *et al.* 2004). The major drawback of the use of this method is ozone short half-life – it decomposes in 20 min – requiring continuous ozonation and making this method expensive to apply (Matsui *in* Reife and Freeman 1996, Slokar and Le Marechal 1998, Robinson *et al.* 2001). Ozone stability is affected by the presence of dyes and salts, by pH and by temperature (Slokar and Le Marechal 1998, Robinson *et al.* 2001). Although thermodynamics for ozone-induced oxidation may be favourable (due to ozone's high reduction potential), kinetic factors will most often dictate whether ozone will oxidize a pollutant in a reasonable time frame (Gogate and Pandit 2004a). It is most useful as a tertiary treatment since the presence of reducing agents and foaming substances, among others, reduces colour removal. Ozone can also be used as a hydrogen peroxide activator (Slokar and Le Marechal 1998). Advantages of its use over the ones referred above are: no residue or sludge formation and no toxic metabolites are formed, and the application in gaseous state not increasing the volume of the wastewater (Robinson *et al.* 2001).

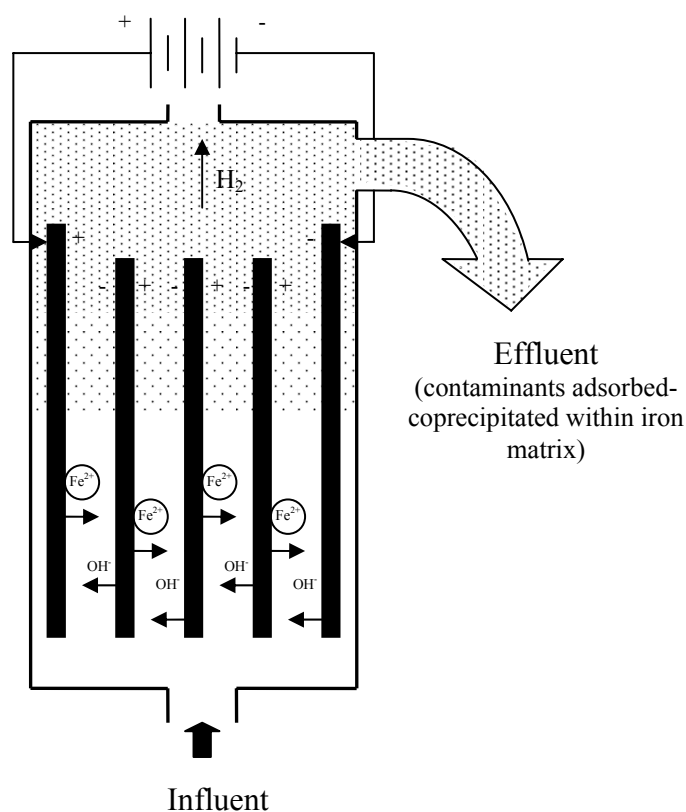
**Photochemical.** Processes like UV/ $\text{H}_2\text{O}_2$ , UV/ $\text{TiO}_2$ , UV/Fenton's reagent, UV/ $\text{O}_3$  and others are photochemical methods based on the formation of free radicals due to UV irradiation. Degradation is caused by the production of high concentrations of hydroxyl radicals and the dye molecule is degraded to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  (Peralta-Zamora *et al.* 1999, Robinson *et al.* 2001, Gogate and Pandit 2004a).

The rate of dye removal is influenced by the intensity of the UV radiation, pH, dye structure and the dye bath composition (Slokar and Le Marechal 1998). When  $\text{H}_2\text{O}_2$  is used as oxidizing agent, the UV light activates the decomposition of  $\text{H}_2\text{O}_2$  into two hydroxyl radicals ( $\text{H}_2\text{O}_2 + h\nu \rightarrow 2\text{OH}\cdot$ ). This method does not produce sludge and greatly reduces foul odours (Robinson *et al.* 2001). There is also the possibility of effectively using sunlight or near UV light for irradiation, which would result in considerable economic savings, especially for large-scale operations (Gogate and Pandit 2004a). Faster, cheaper and more effective photocatalytic processes are based on catalysis by solid semiconductor materials, mostly  $\text{TiO}_2$  particles (van der Zee 2002, Konstantinou and Albanis 2004). With  $\text{TiO}_2$  catalysed UV treatment a wide range of dyes can be mineralised (Gonçalves *et al.* 1999, Peralta-Zamora *et al.* 1999, de Moraes *et al.* 2000, Bauer *et al.* 2001, Augugliaro *et al.* 2002, da Silva and Faria 2003). The photodegradation of dyes by this method depends considerably on the chemical structure of the dye (Forgacs *et al.* 2004).

**Sodium hypochlorite ( $\text{NaOCl}$ ).** Coloured wastewaters can be chemically oxidized by chlorine compounds although, for environmental reasons (release of toxic organochlorinated compounds), its use is becoming less frequent (Slokar and Le Marechal 1998, Pizzolato *et al.* 2002). Electrophilic attack at the amino group by  $\text{Cl}^+$  initiates and accelerates the subsequent azo bond cleavage. The decolourisation is affected by pH and by  $\text{NaOCl}$  concentration (Slokar and Le Marechal 1998, Robinson *et al.* 2001). This method is not efficient for the decolourisation of disperse dyes, and longer times are required to decolourise reactive and metal-complex dyes (Slokar and Le Marechal 1998).

**Electrolysis.** Electrolysis (see Figure 8) is based on applying an electric current through to the wastewater by using electrodes. Organic compounds like dyes react through a combination of electrochemical oxidation, electrochemical reduction, electrocoagulation and electroflotation reactions. For instance, when iron is the sacrificial anode,  $\text{Fe(II)}$ -ions are released to the bulk solution, and acid dyes are sorbed on the precipitated  $\text{Fe(OH)}_2$ . Moreover  $\text{Fe(II)}$  can reduce azo dyes to arylamines (Vandevivere *et al.* 1998, Wilcock *et al.* in Reife and Freeman 1996). Moreover, water can also be oxidized resulting in the formation of  $\text{O}_2$  and  $\text{O}_3$  and, if chloride is present, there is also formation of  $\text{Cl}_2$  and oxychloride anions. In the cathode occurs reduction of water to  $\text{H}_2$  and  $\text{OH}^-$ . In order to improve the performance of the system different materials have been tested in the electrodes like carbon-fibre,  $\text{Ti/Pt}$  and aluminium (Vlyssides *et al.* 1999, Shen *et al.* 2001, Kobya *et al.* 2003, Cerón-Rivera *et al.* 2004, Fernandes *et al.* 2004). The main disadvantage of these types of methods is the cost, both initial capital costs, energy and of electrode replacement (Vandevivere *et al.* 1998, van der Zee 2002, Kobya *et al.* 2003, Cerón-Rivera *et al.* 2004). The formation of unwanted breakdown products and

foam are also drawbacks of this method (Vandevivere *et al.* 1998, van der Zee 2002). The main advantages are compact size of equipment, simplicity in operation, fast rate of pollutant removal and decrease amount of sludge generated (Gürses *et al.* 2002, Daneshvar *et al.* 2004). The method is efficient for colour, BOD (biochemical oxygen demand), COD (chemical oxygen demand), TOC (total organic carbon), TDS (total dissolved solids), TSS (total suspended solids) and heavy metals removal (Wilcock *et al.* in Reife and Freeman 1996, Kobya *et al.* 2003, Daneshvar *et al.* 2004, Fernandes *et al.* 2004).



**Figure 8:** Illustration of an electrochemical cell (adapted from Wilcock *et al.* in Reife and Freeman 1996).

**Wet air oxidation (WAO).** In this process the primary oxidant species is oxygen and it is used to produce OH-radicals which actually react with organic and inorganic species under sub-critical conditions, i.e. temperature between 175 and 320°C and pressure between 60 and 200 bar (Heimbuch and Wilhelmi 1985, Jeworski and Heinzle 2000, Arslan-Alaton and Ferry 2002, Gogate and Pandit 2004b). These operation conditions result in high capital investments and high maintenance costs (Arslan-Alaton and Ferry 2002). Economic operation of WAO processes requires wastewaters with COD contents above 20g.L<sup>-1</sup> to allow autothermic operation (Jeworski and Heinzle 2000).

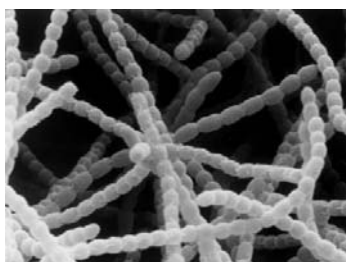
**Ultrasound.** This technology can also be used in textile dye remediation but is still a very recent technology in phase of development (Gogate and Pandit 2004a, Vajnhandl and Le Marechal 2005).

### 1.6.3 BIOLOGICAL TREATMENTS

The fate of environment pollutants is largely determined by abiotic processes, such as photooxidation, and by the metabolic activities of microorganisms. Since catabolic enzymes are more or less specific, they can act on more than their natural substrate. This explains why the majority of xenobiotics are subject to fortuitous metabolism (cometabolism) (Knackmuss 1996) and several groups explore these microbial capacities for the bioremediation of dyes.

The limitations of biological processes are mainly caused by limited biodegradability of primarily xenobiotic compounds like dyes, by toxic or inhibitory effects of pollutants for the microbial population and by the slow rate of biodegradation of particular pollutants (Jeworski and Heinzle 2000).

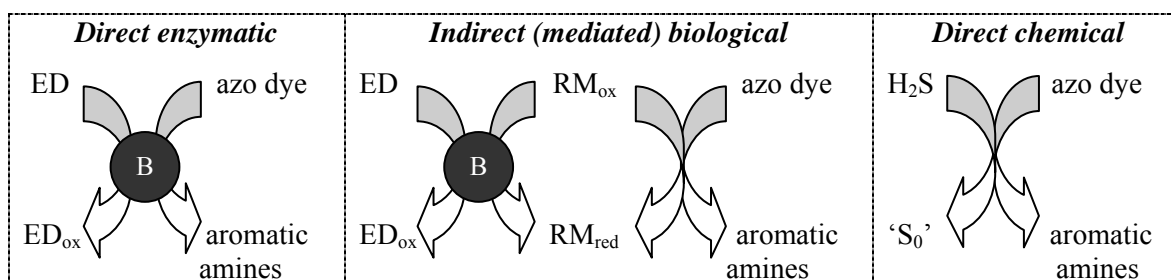
**Bacteria.** Actinomycetes, particularly *Streptomyces* species (Figure 9), are known to produce extracellular peroxidases that have a role in the degradation of lignin and were also found effective in the degradation of dyes (Paszczyński *et al.* 1991, Zhou and Zimmerman 1993, Cao *et al.* 1993, McMullan *et al.* 2001, Bhaskar *et al.* 2003).



**Figure 9.** Microscope photography of *Streptomyces* spp.

In these studies were used azo dyes with exception for Zhou and Zimmerman that tried reactive dyes (anthraquinone, phthalocyanine, azo and metal complex dyes) (Zhou and Zimmerman 1993). With the notable exception of actinomycetes, the isolation of bacteria capable of aerobic decolourisation and mineralization of dyes, specially sulfonated azo dyes, has proven difficult (McMullan *et al.* 2001). For aerobic bacteria to be significant in the reductive process they must be specifically adapted (Pearce *et al.* 2003). Using this methodology, and testing some analogues of sulfonated azo dyes as sole source of carbon and energy, several groups manage to isolate and purify “azoreductases” from *Pseudomonas* strains KF46 (Zimmermann *et al.* 1982) and K24 (Zimmermann *et al.* 1984) and from *Xenophilus azovorans* KF46F (Blümel *et al.* 2002). The ability of bacteria to aerobically metabolise other dye classes was described for *Kurthia* sp. (Sani and Banerjee 1999) and for *Pseudomonas mendocina* MCM B-402 (Sarnaik and Kanekar 1999).

Some possible models for the anaerobic azo dye reduction are shown in Figure 10.



**Figure 10.** Mechanisms of anaerobic azo dye reduction by bacteria ( $RM_{ox}$  = oxidised redox mediator;  $RM_{red}$  = reduced redox mediator; ED = electron donor;  $ED_{ox}$  = oxidised electron donor; B = bacteria (enzyme system)) (adapted from van der Zee 2002).

The initial step in bacterial azo dye metabolism under anaerobic conditions involves the reductive cleavage of the azo linkage. This process is catalysed by a variety of soluble cytoplasmic enzymes with low-substrate specificity, which are known as “azoreductases” (Robinson *et al.* 2001, Stolz 2001). Under anoxic conditions, these enzymes facilitate the transfer of electrons via soluble flavins to the azo dye, which is then reduced. The role that such cytoplasmic enzymes have *in vivo* is, however, uncertain (McMullan *et al.* 2001). The work of Russ and co-workers showed however that the cytoplasmic “azoreductases” are presumably flavin reductases and that they have insignificant importance in the *in vivo* reduction of sulfonated azo compounds (Russ *et al.* 2000). The possibility of non-cytoplasmic azoreductases is then reinforced since it is highly improbable that highly charged sulfonated azo dyes or polymeric azo dyes pass through the bacterial cell wall (Keck *et al.* 1997). A membrane bound “azoreductase” was found by Kudlich and co-workers in the cell wall of a *Sphingomonas* sp. This strain possessed both cytoplasmic and membrane-bound azoreductase activities (Kudlich *et al.* 1997). A different model for the unspecific reduction of azo dyes by bacteria was proposed based on studies with *Sphingomonas xenophaga* BN6 (Keck *et al.* 1997). They observed the increase in the reduction rate when quinones, like anthraquinone-2-sulfonate or 2-hydroxy-1,4-naphthoquinone, were added to the culture medium. It was suggested then that quinones added to the medium or some decomposition products released by the cells to the medium, acted as redox mediators which were enzymatically reduced by the bacteria cells and that the hydroquinones formed reduced the azo dye in a purely chemical redox reaction. Rafii suggests an extracellular azoreductase activity in studies done with bacteria isolated from human intestine, mainly *Eubacterium* sp. and *Clostridium* sp. (Rafii *et al.* 1990, Rafii *et al.* 1995). Another possibility for the extracellular reduction of azo compounds under anaerobic conditions is the action of reduced inorganic compounds (e. g.  $Fe^{2+}$ ,  $H_2S$ ), that are formed as end products of certain strictly anaerobic bacterial metabolic reactions (Stolz 2001, van der Zee *et al.* 2001, Yoo 2002, van der Zee *et al.* 2003). The further mineralization of the

formed amines is not possible under anaerobic conditions (Brown and Hamburger 1987). This is why there are several studies that propose a combined anaerobic-aerobic system for the removal of dyes from wastewaters with a consortium/sludge (Haug *et al.* 1991, Seshadri *et al.* 1994, O'Neill *et al.* 2000, Lourenço *et al.* 2001, Fu *et al.* 2002, Shaw *et al.* 2002, Sponza and Isik 2002, Isik and Sponza 2004, Libra *et al.* 2004, Supaka *et al.* 2004, Sponza and Isik 2005). The utilization of consortia offers considerable advantages over the use of pure cultures in the degradation of synthetic dyes. The individual strains may attack the dye molecule at different positions or may use the decomposition products produced by another strain for further decomposition. However, the composition of mixed cultures may change during the decomposition process interfering with the control of the system. The most used consortium is activated sludge system, mainly constituted by bacteria, but also with the usual presence of fungi and protozoa.

**Fungi.** The most widely studied dye-decolourising microorganisms are the white-rot fungi like *Phanerochaete chrysosporium* (Figure 11) (Glenn and Gold 1983, Bumpus and Brock 1988, Pasti-Grigsby *et al.* 1992, Chao and Lee 1994, Conneely *et al.* 1999, Chagas and Durrant 2001, Kunz *et al.* 2001, Martins *et al.* 2002, Mielgo *et al.* 2002), *Trametes versicolor* (Field *et al.* 1992, Borchert and Libra 2001, Tekere *et al.* 2001), *Coriolus versicolor* (Kapdan *et al.* 2000, Kapdan and Kargi 2002) and *Bjerkandera adusta* (Field *et al.* 1992, Heinfling-Weidtmann *et al.* 2001).



**Figure 11:** White-rot fungi *Phanerochaete chrysosporium* in its natural environment, wood.

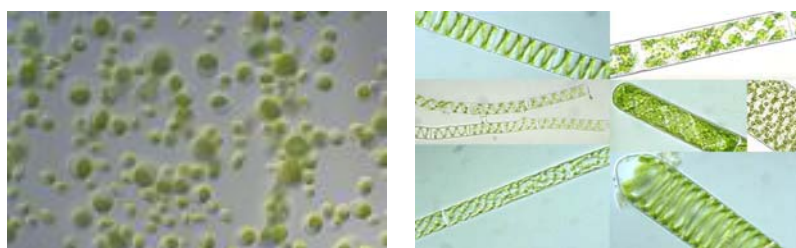
Several other non-white-rot fungi can also successfully decolorize dyes like *Aspergillus niger* (Abd El-Rahim and Moawad 2003), *Geotrichum candidum* (Kim *et al.* 1995, Kim and Shoda 1999), *Pleurotus ostreatus* (Martins *et al.* 2003, Palmieri *et al.* 2005) and *Cunninghamella elegans* (Cha *et al.* 2001, Ambrósio and Campos-Takaki 2004) among others (Fu and Viraraghavan 2001). White-rot fungi constitute a diverse ecophysiological group comprising mostly basidiomycetous fungi capable of aerobic lignin depolymerization and mineralization, playing a central role in the global C-cycle (McMullan *et al.* 2001, Wesenberg *et al.* 2003). This ability is correlated to the capacity of these organisms to synthesise lignin-degrading extracellular enzymes such as lignin peroxidases (LiP) and manganese peroxidases (MnP), or laccases (Lac) (Robinson *et al.* 2001, Stolz 2001, Forgacs *et al.* 2004) which, thanks to



their lack of substrate specificity, are also capable of degrading a wide range of xenobiotics (Reddy 1995, McMullan *et al.* 2001, van der Zee 2002, Wesenberg *et al.* 2003, Novotný *et al.* 2004). Among these are dioxins, polychlorinated biphenyls (PCBs), chlorophenols, polycyclic aromatic hydrocarbons (PAHs) and nitroaromatics, including dyes (Reddy 1995, Robinson *et al.* 2001, Wesenberg *et al.* 2003, Forgacs *et al.* 2004). LiP catalyses the oxidation of non-phenolic aromatic compounds such as veratryl alcohol. MnP oxidizes preferably  $Mn^{2+}$  to  $Mn^{3+}$  which is able to oxidise many phenolic compounds. Laccase is a copper-containing enzyme that catalyses the oxidation of phenolic substrates by coupling it to the reduction of oxygen to water (McMullan *et al.* 2001, Wesenberg *et al.* 2003). Whilst it is clear that these enzymes play a significant role in dye metabolism, care must be taken not to exclude the possibility of the existence of other degradative mechanism (McMullan *et al.* 2001). Recently a third group of peroxidases, versatile peroxidase (VP), has been recognized in species of *Pleurotus* and *Bjerkandera*, (Heinfling *et al.* 1998 a, Heinfling *et al.* 1998 b). A number of other enzymes are produced in parallel including  $H_2O_2$ -producing enzymes required by other peroxidases (glyoxal oxidase and superoxide dismutase), and enzymes linked to lignocellulose degradation pathways (glucose oxidase and aryl alcohol oxidase) (Wesenberg *et al.* 2003, Novotný *et al.* 2004). Although these fungi have been shown to decolourise dyes in liquid fermentations, enzyme production has also shown to be unreliable mainly due the unfamiliar water environment (Robinson *et al.* 2001). Their performance is also closely related to the operation conditions (concentration of dye, pH and temperature), which is a serious drawback for this type of wastewaters (Fu and Viraraghavan 2001). Nevertheless they have the potential to oxidise substrates that have low solubility which is an advantage for the treatment of non-soluble dyes (like vat for instance). Another advantage of these systems is that the constitutive nature of the enzymes obviates the need for the adaptation (Reddy 1995). In process design and optimization of fungal treatment there are some features that should be considered. As the decolourization of dyes by *P. chrysosporium* and other white-rot fungi occurs in secondary metabolic conditions, the important enzyme system is released by the fungal cells under either carbon or nitrogen limitation (Glenn and Gold 1983, Spadaro *et al.* 1992, Wesenberg *et al.* 2003). Production of LiP and MnP is generally optimal at high oxygen tension but is repressed by agitation in submerged liquid culture, while laccase production is often enhanced by agitation. Usually more than one isoform of the enzyme system is expressed by different taxa and culture conditions (Wesenberg *et al.* 2003). Due to the complexity of these systems, both dye structures and enzymatic transformations involved, there is a gap in the knowledge of the degradation and mineralization of dyes by these microorganisms.

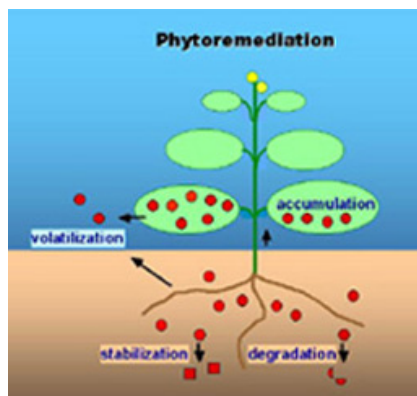
**Yeasts.** In literature the ability to degrade azo dyes by yeasts was only described in a few reports. The first two reports use the ascomycete yeast *Candida zeylanoides* isolated from contaminated soil to reduce model azo dyes (Martins *et al.* 1999, Ramalho *et al.* 2002). The characterisation of an enzymatic activity is described in further studies with the yeast *Issatchenkia occidentalis* (Ramalho *et al.* 2004), and the enzymatic system involved is presented in a work with *Saccharomyces cerevisiae* (Ramalho *et al.* 2005).

**Algae.** The use of algae for the degradation of dyes is mentioned in only few reports and is achieved by *Chlorella* (Jinqi and Houtian 1992, Acuner and Dilek 2004), *Oscillatoria* (Jinqi and Houtian 1992) and *Spirogyra* (Mohan *et al.* 2002) species (Figure 12). All these reports used azo dyes. Jinqi and Houtian (Jinqi and Houtian 1992) and Acuner and Dilek (Acuner and Dilek 2004) mention the reduction of the azo bond as the decolourising mechanism. Mohan (Mohan *et al.* 2002) attributes the decolourisation to biosorption followed by bioconversion and biocoagulation. Furthermore they all state that the formed amines can be totally mineralized. Jinqi and Houtian (Jinqi and Houtian 1992) also state that some of the tested azo compounds could be used as sole sources of carbon and nitrogen by the algae. This could mean that algae can play an important role in the removal of azo dyes and aromatic amines in stabilization ponds (Banat *et al.* 1996).



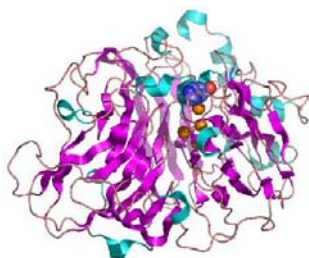
**Figure 12:** Microscope images of *Chlorella vulgaris* and *Spirogyra* spp.

**Plants.** There are two recent studies that describe the use of plants (phytoremediation – Figure 13) for the dye removal from wastewaters. The first one mentions a good removal capacity of sulphonated anthraquinones with *Rheum rabarbarum* (Aubert and Schwitzguébel 2004), although only shows preliminary results that need to be further investigated. This plant possesses enzymes that accept anthraquinones as substrates and in cell culture were able to remove up to  $700\text{--}800\text{mg.L}^{-1}$  of anthraquinones with sulphonate groups in different positions. Also Mbuligwe describes a reduction in colour of 72-77% in wetlands vegetated with coco yam plants (Mbuligwe 2005). These systems have the serious disadvantage of requiring big areas to implant the treatment.



**Figure 13:** Process of phytoremediation.

**Enzymes.** In the studies of biological degradation of dyes an effort as been made in order to identify, isolate and test the enzymes responsible for the decolourisation. In the case of extracellular fungal enzymes, like manganese and lignin peroxidases and laccases (Figure 14), or cytosolic azoreductases from bacteria, this has been achieved by several groups (Dass and Reddy 1990, Gosh *et al.* 1992, Spadaro and Renganathan 1994, Heinfling *et al.* 1998a, Rafii and Coleman 1999, Schliephake *et al.* 2000, Campos *et al.* 2001, Suzuki *et al.* 2001, Nyanhongo *et al.* 2002, Blümel and Stolz 2003, Ryan *et al.* 2003, Maier *et al.* 2004).



**Figure 14:** Laccase from *Trametes versicolor*.

The application of enzyme preparations shows considerable benefits over the use of microorganisms. Commercial preparations can be easily standardized, facilitating accurate dosage. The application is simple and can be rapidly modified according to the character of the dye or dyes to be removed (Forgacs *et al.* 2004). Nevertheless the use of whole cells rather than isolated enzymes is advantageous, because costs of purification are extremely high and the cell offers protection from the harsh process environment to the enzymes. Also, degrading is often carried out by a number of enzymes working sequentially and not by one single enzyme (Pearce *et al.* 2003).

## **CHAPTER 2**

### **REDUCTIVE DECOLOURISATION OF AZO DYES BY *CANDIDA ZEYLANOIDES***

# Improved conditions for the aerobic reductive decolourisation of azo dyes by *Candida zeylanoides*

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## Abstract

A number of anaerobic and aerobic bacterial species are known to decolourise azo dyes through the reduction of the azo bonds, forming the corresponding amines. In this work, we describe improved decolourisation conditions for model azo dyes by the ascomycete yeast *Candida zeylanoides*. The dyes were derived from the diazonium salts of metanilic and sulfanilic acids and *N,N*-dimethylaniline or 2-naphthol as coupling components. Total decolourisation times observed in culture media supplemented with 0.2mM dye ranged from 40 to 60 h. The initial decolourisation rates were 14–52  $\mu\text{mol (g dry cell)}^{-1} \text{ h}^{-1}$ , depending on dye structure. In the course of decolourisation either metanilic acid or sulfanilic acid were detected in the supernatant fluid, showing that decolourization by this yeast strain is due to azo bond reduction. None of those aminobenzenesulfonates supported microorganism growth as carbon and energy source but both could be used, to a limited extent, as nitrogen sources. The azo reductase activity is not significantly affected by pre-adaptation of the microorganism to the dyes.

**Keywords:** Azo dyes; Methyl orange; Orange II; Yeasts; Decolourisation

## 1. Introduction

Among the synthetic dyes, which are widely used for textile dyeing and other industrial applications, those containing an azo chromophore constitute the largest class [1]. Since dyes are designed to be resistant to microbial and physico-chemical attack, most of them are not easily destroyed by conventional processes of wastewater treatment, including biological treatment by activated sludge [2,3]. The percentage of unchanged dye after such treatments is estimated to range between 50 and 90% [4,5], a fact which raises environmental problems, not only for obvious aesthetic reasons but also because the dyes themselves, or their biotransformation products, can have deleterious effects on living organisms. Both the structural diversity of dyes and the wide variability in composition of dye-containing effluents are probably decisive factors accounting for the observed recalcitrance of colouring substances [6].

As reviewed by Chung et al. [7], earlier studies on the decolourisation of azo dyes mainly involved anaerobic bacterial species isolated from the intestinal microflora, which cleave the molecules by reduction of the azo bond(s). A closely related

research field deals with the investigations on the toxic, mutagenic, or carcinogenic properties of several aromatic amines generated by biological reduction [8,9]. The azo reductase activity detected in bacteria displaying azo bond reduction capabilities has also deserved considerable attention. Azo reductases were isolated from several bacterial sources and some of their molecular properties and substrate specificities were determined [10,11]. Some authors have also succeeded in isolating genomic DNA fragments from azo-reducing bacterial species and expressing them in non-decolourising species [12–14]. The available evidence indicates that azo reductase activity is associated with different types of genes [12]. In other words, azo reductase activity can be associated with more than one reductase, depending on the particular microorganism and, eventually, on the cultivation conditions.

The most generally accepted mechanism of azo reduction involves the participation of redox mediators, acting as electron shuttles between the extracellular dye and the intracellular reductase. The azo bond reduction step thus occurs extracellularly. Anthrahydroquinone sulfonates [15,16], riboflavin and benzylviologen [17] are examples of redox mediators which have been shown to accelerate azo bond reduction by intact cells. This mechanism also accounts for the reduction of highly polar azo dyes, which can not usually cross cell membranes.

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Bacterial azo bond reduction also occurs under aerobic conditions, as demonstrated, about two decades ago [18,19]. Since then various reports on anaerobic, aerobic or anaerobic–aerobic sequential treatments for degradation of coloured effluents have appeared, as recently reviewed by Robinson et al. [20] and Stolz [21].

In an earlier report [22], we have provided preliminary evidence on the aerobic azo dye decolourisation by an ascomycete yeast, identified as *Candida zeylanoides*. In the present work, we have used modified cultivation conditions in order to improve the decolourisation process by the same microorganism, and investigated some features of the azo reductase activity which mediates dye decolourisation. The utilization of the stable reduction products of the tested dyes by the microorganism was also studied.

## 2. Materials and methods

### 2.1. Chemicals and culture media components

The structures of the azo dyes used in this work are represented in Table 1. Dyes I–III were synthesized and characterized by conventional methods. Dye IV was purchased from Sigma-Aldrich and used without further purification. Inorganic media components, D-glucose, 3,5-dinitrosalicylic acid (DNS) and methanol, of analytical grade, were obtained from Merck. Tetrabutylammonium phosphate (TBAP), sulfanilic acid and metanilic acid were from Sigma-Aldrich. Complex media components (yeast extract, yeast carbonbase (YCB), yeast nitrogen base (YNB) and peptone) were obtained from Difco.

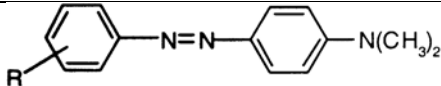
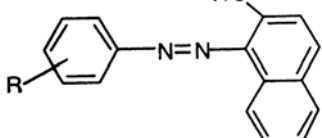
### 2.2. Microorganism and maintenance conditions

The ascomycete yeast *C. zeylanoides* (strain UM2), was isolated as described in a previous publication [22]. The strain was routinely maintained on slants of a medium containing (w/v, %): glucose (2), peptone (1), yeast extract (0.5), and agar (2).

### 2.3. Analytical methods

Biomass was measured by turbidimetry readings of appropriately diluted culture samples at 640 nm, against a blank prepared with the same dilution of the supernatant in distilled water. The correlation between OD (640 nm) and cell dry weight ( $w$ ,  $\text{g l}^{-1}$ ) was  $\text{OD} = 1.40 w - 0.14$  ( $r^2 = 0.996$ ), as experimentally determined by the standard gravimetric method. Dye concentration was estimated by absorbance measurements of supernatant samples, diluted as required, in a buffer of pH 4.0, at dye  $\lambda_{\text{max}}$ , read against a blank containing the same dilution of buffer in water. Glucose concentration was determined with the DNS reagent [23]. HPLC analyses for detection of dye metabolites were performed in a liquid chromatograph consisting of a pump (model PU-980) and a UV/VIS detector (model UV-975), both from Jasco, and a Chromatopac C-R6A recorder, from Shimadzu. The column (Lichrocart 250-4) was packed with Lichrospher-100 RP-18, 5  $\mu\text{m}$ , from Merck. Sample injection was performed with a 20  $\mu\text{l}$  Rheodyne injector. The eluent composition was 30:70 (v/v) methanol–water, containing 5mM TBAP. The flow rate was adjusted to 0.6  $\text{ml min}^{-1}$  and the separated compounds were detected at 220

**Table 1.**  
Structures and  $\lambda_{\text{max}}$  of dyes

Parent structure	Dye	$\lambda_{\text{max}}$ (nm)
	I: R = <i>m</i> -SO <sub>3</sub> Na <sup>a</sup>	461
	II: R = <i>p</i> -SO <sub>3</sub> Na <sup>b</sup>	470
	III: R = <i>m</i> -SO <sub>3</sub> Na <sup>c</sup>	481
	IV: R = <i>p</i> -SO <sub>3</sub> Na <sup>d</sup>	484

<sup>a</sup> *m*-[(4-Dimethylamino)phenylazo]benzenesulfonic acid, sodium salt.

<sup>b</sup> *p*-[(4-Dimethylamino)phenylazo]benzenesulfonic acid, sodium salt (Methyl Orange/Orange 52, C.I. 13025).

<sup>c</sup> *m*-[(2-Hydroxy-1-naphthyl)azo]benzenesulfonic acid, sodium salt.

<sup>d</sup> *p*-[(2-Hydroxy-1-naphthyl)azo]benzenesulfonic acid, sodium salt (Orange II/Acid Orange 7, C.I. 15510).

nm. Under these conditions the markers for azo bond reduction of Dyes I/III and II/IV, metanilic acid and sulfanilic acid, respectively, had retention times of  $12.8 \pm 0.3$  and  $10.3 \pm 0.3$  min. The presence of these compounds in the supernatant fluids was confirmed by using authentic samples as internal standards. Glucose, ethanol and acetic acid concentrations were occasionally determined in the growth media by HPLC, using a Merck Polyspher OA KC column, with refractive index detection, and arabinose as internal standard [24].

#### 2.4. Growth and decolourisation assays

Typical decolourisation experiments were routinely performed in 250 ml conical flasks with 100 ml volumes of a sterile medium (normal decolorization medium, NDM) containing (w/v, %):  $(\text{NH}_4)_2\text{SO}_4$  (0.25), yeast extract (0.25),  $\text{KH}_2\text{PO}_4$  (0.5),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.05),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.013) and glucose (2). Concentrated azo dye solutions (4 mM) were separately prepared and filter sterilized (Filtropur  $0.2\mu\text{m}$ ) and aseptically added to the medium to a final concentration of 0.2 mM. The flasks were inoculated with a cell suspension obtained from a freshly grown slant, and incubated at  $25^\circ\text{C}$ , under orbital shaking (120 rpm). Triplicate experiments were run throughout. In order to elucidate if pre-adaptation of the microorganism to the dyes had any effect on decolorization rates, cultures were started at a high initial cell density (ca. 1.3 OD units;  $1.0 \text{ g dry cell l}^{-1}$ ). The inocula were prepared from cells in the exponential growth phase, obtained after ca. 14 h incubation, in the presence or in the absence of dye, in 100 ml volumes of NDM. Adapted and non-adapted cells were harvested by centrifugation and washed twice before resuspension in 100 ml volumes of fresh NDM, containing 0.2 mM dye.

#### 2.5. Assimilation experiments

Minimal media (YNB, and YCB) were prepared according to the manufacturer's instructions and supplemented with 5 mM metanilic or sulfanilic acid for testing these compounds as carbon and energy sources or nitrogen sources for the microorganism. Control experiments were performed with 2.5 mM ammonium sulfate as sole nitrogen source, in YCB, or with 5 mM glucose as sole carbon and energy source, in YNB.

### 3. Results and discussion

#### 3.1. Dye effects on the microorganism specific growth rates

In order to detect possible dye inhibitory effects, separate growth experiments in NDM, containing each one of the Dyes I–IV, and a control without dye, were run. As shown in Fig. 1, very similar growth curves were obtained. The observed duplication times ranged between 4.8 and 5.6 h, and the biomass, in the stationary phase, reached similar values (5–6 OD units) at 40 h incubation. These results indicate that, at the concentrations used none of the tested dyes had a significant inhibitory effect on the microorganism growth.

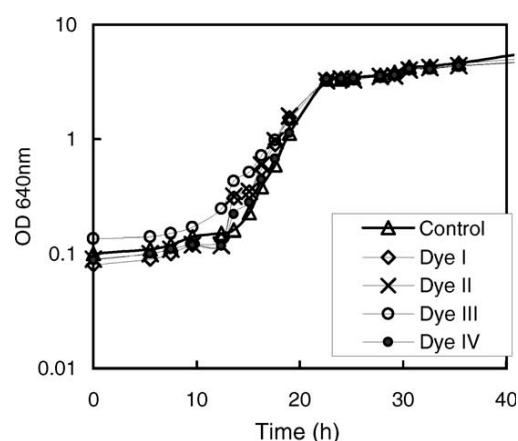


Fig. 1. Growth curves of *C. zeylanoides* in NDM containing 0.2 mM Dyes I–IV and control growth curve in the same medium without dye.

#### 3.2. Decolourisation experiments

As described in a previous report [22], between 10 and 50% of Dyes I–IV (formerly Dyes 2b, 2a, 1b and 1a) persisted in the supernatants of shaking cultures of *C. zeylanoides*, after 7 days (168 h) of incubation. By using the new culture medium (NDM) it could consistently be observed that, after 40 h incubation, the concentration of Dyes I and II was undetectable, and that of Dyes III and IV was reduced to ca. 15% (Fig. 2). The incubation period required for complete decolourisation of the latter dyes was 60 h (results not shown). In any case, when decolourisation was complete, the yeast cells were also colourless, indicating the absence of any adsorption phenomenon. These results reflect a considerable decrease in the period required for complete decolourisation of these dyes by *C. zeylanoides*, under conditions that, except for the

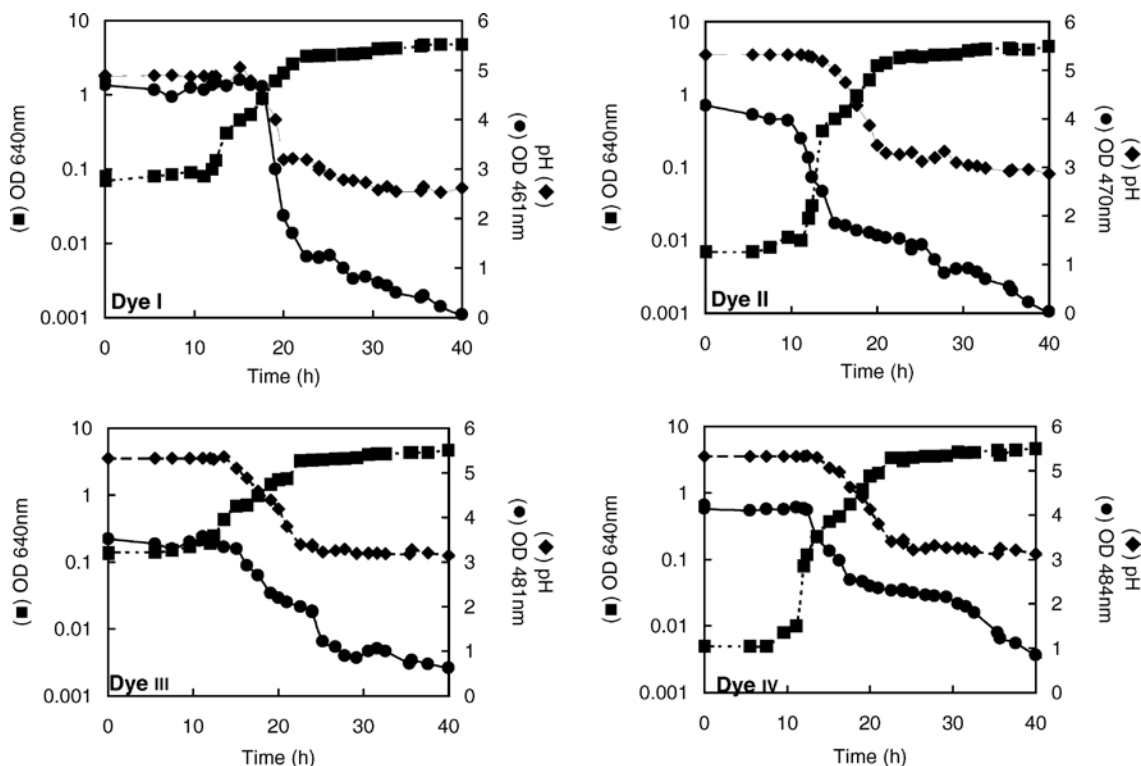


Fig. 2. Time-course of the decolourisation of Dyes I-IV (●), evolution of pH (▲) and cell growth (■) in batch aerated cultures of *C. zeylanoides*.

medium composition, were otherwise similar.

From Fig. 2, it is also apparent that decolourisation is faster during the exponential growth phase of the microorganism, slowing down in the stationary phase. This is not unexpected, since the process must be dependent on cell biomass and on actively growing cells.

The monitoring of pH revealed a progressive acidification of the culture medium, starting at the onset of the exponential growth phase, which is due to glucose metabolism. Under the conditions described, the presence of ethanol and acetic acid was detected in samples of supernatant fluid collected after ca. 20 and 25 h incubation, respectively (results not shown). The observed pH variation can, eventually, affect decolourisation rate, as is usually the case for bacterial decolourisation processes [17,25], and also for the chemical azo dye reduction by NADH [26]. However, further experimental evidence will be required in order to draw any conclusion regarding the optimum pH range for the decolorization process by this yeast strain.

### 3.3. Detection of dye reduction products

HPLC analyses of supernatant samples of dye decolourisation cultures revealed the presence of metanilic acid (for Dyes I and III), or sulfanilic acid (for Dyes II and IV), as illustrated in Fig. 3. These aminobenzenesulfonates are the stable reduction products of the tested dyes and were therefore used as markers for azo bond reduction. The other expected reduction products, 1-amino-2-naphthol and *N,N*-dimethyl-*p*-phenylenediamine, are quite unstable in aerated solutions. This behaviour has already been described by Kudlich et al. [27] for 1-amino-2-naphthol. The results described above demonstrate that the yeast strain used in the present work cleaves azo bonds by reduction, forming the corresponding amines, as observed with decolourising anaerobic or facultative aerobic bacteria.

### 3.4. Assimilation experiments

Metanilic and sulfanilic acids were separately tested as carbon and energy sources or nitrogen sources by determining specific growth rates of the yeast cells in minimal media (YNB or YCB) supplemented with those compounds, at 5mM concentrations. Appropriate controls, as described



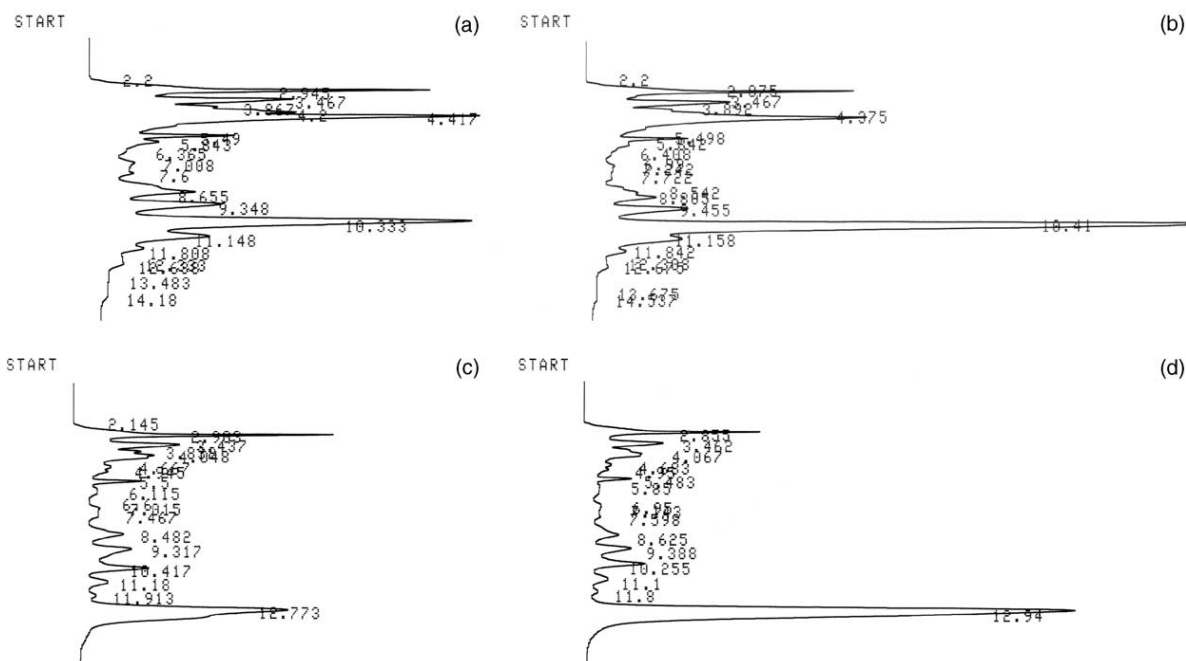


Fig. 3. Chromatograms of supernatant samples taken from growing cultures in media containing Dyes II and III (a and c); chromatograms of the same samples with added sulfanilic and metanilic acids, respectively (b and d).

in Section 2, were simultaneously run. The results displayed in Fig. 4 show that the two aminobenzenesulfonic acids behave similarly as nitrogen sources, with duplication times of 17 h, much higher than the observed with equimolar inorganic ammonium (6.7 h). The limited growth observed in YCB can be due to a restricted uptake of the nitrogen sources by the yeast cells or, alternatively, to the formation of some inhibitory product. This aspect is currently being investigated.

In contrast, none of those substrates supported microorganism growth, as carbon and energy source. The sulfonic group occurs rarely in nature and, therefore, degradation mechanisms for sulfonates are not frequent in the microbial world [28]. There are, however, some examples described in the literature relating the mineralization of naphthalenesulfonated by a bacterial consortium [29] and of sulfanilic acid by a single bacterial strain, obtained from a mixed culture after extended preadaptation [30]. A similar capability has not been described, so far, in yeast species.

### 3.5. Effect of cell pre-adaptation on decolourisation rates

In order to obtain some evidence regarding the constitutive or inducible nature of the azo reductase activity, the decolourisation rates by cells pre-adapted to the dyes were compared with those observed in cultures inoculated with cells grown in

a medium without dye but, otherwise, of identical composition. The results displayed in Fig. 5 clearly show that azo bond reduction is mediated by some constitutive reductase since, in every case, the decolorization progress is virtually independent on cell pre-adaptation. The observed differences in the half decolourisation times, which range from 1.5 to 4.5 h, are therefore related to dye structure.

### 3.6. Structural effects on decolourisation rates

Azo dye reduction in the presence of intact cells, by a redox shuttle mechanism, is expected to be an apparent zero-order reaction, while cells are actively growing, and reduced cofactors are abundant. This seems to be the case with the yeast strain used in this work. In fact, following a short latency phase, observed with Dyes I, III and IV, dye concentration decreases linearly with time, at least in the first half of the decolorization process (Fig. 5). The apparent decolourisation rates for Dyes I–IV, estimated in the linear portion of the decolourisation curves, and referred to initial dry cell weight, are shown in Table 2.

Table 2. Apparent decolorization rates

Dye	Rate ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ )
I	23
II	52
III	18
IV	14

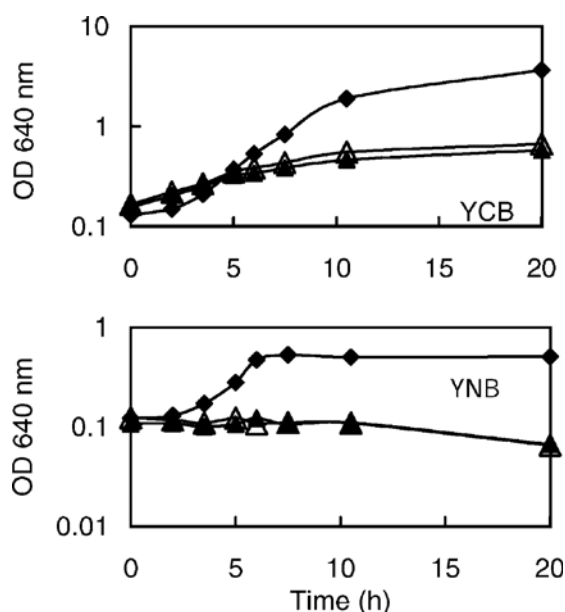


Fig. 4. Growth curves obtained in YCB and YNB containing 5mM metanilic acid (▲) or 5mM sulfanilic acid (■); controls (◆) were done in the presence of 2.5mM ammonium sulfate (in YCB) or 5mM glucose (in YNB).

Interestingly Dyes I, III and IV have very similar apparent zero-order decolorization rates, but significantly lower than the observed with Dye II. These results are, so far, intriguing, even taking into account some of the factors which are likely to affect decolourisation rates, as the steric and electronic characteristics of the substrate, the redox potential of the mediator in relation to that of the azo compound, or the specificity of the reductase towards the mediator. Indeed, in Dyes III and IV, which share the same coupling component (2-naphthol), the position of the sulfonic substituent in the diazo component does not significantly affect the decolorization rate. In contrast, in Dyes I and II, where the only structural difference is also the position of the sulfonic substituent, those rates are rather different. One hypothesis which could account for the (so far) unexplained differences in decolourisation rates is the occurrence of distinct reduction mechanisms, depending on the particular dye substrate. Two possible mechanisms for complete azo reduction are a NAD(P)H-like reduction, involving two steps of hydride transfer, each preceded (or followed) by protonation or, alternatively, a flavin-like reduction, where four steps of  $H^+ + e^-$  addition would take place. Quite likely the limiting rates in each mechanism would be differently affected by structural features of the substrates. This hypothesis might also account for other unexplained observations as those of Semdé

et al. [17] who reported that a NADPH generator system, while being able to reduce the dye amaranth, did not reduce methyl orange or orange II (Dyes II and IV in this study), among other dyes. Also Nam and Renganathan [26], in their study of the non-enzymatic reduction of azo dyes by NADH, were not able to establish a simple correlation between the electronic properties of the substituents in 4-(4'-sulfophenylazo)phenol derivatives, or their position relatively to the azo bond, and the decolourisation extent, after a fixed time. It thus appears that a suitable basis for correlating azo reduction rates and dye structures has not yet been found.

#### 4. Conclusions

The present work described improved conditions for the decolourisation of several structurally-related azo dyes by a strain of the yeast *C. zeylanoides*. Apparently, the major factor determining the shorter decolourisation times now described is the composition of the incubation medium. Decolourisation is due to azo bond reduction, as shown by the detection of the corresponding amines in the supernatants of cultures where dye decolorization was taking place, and occurs at an acidic pH. The sulfonated amines produced by reduction of the tested dyes could be used as nitrogen sources, but not as carbon and energy sources by the yeast strain. The azo reductase activity towards each dye does not depend on pre-adaptation of the cells to that dye and is therefore due to an unidentified reductase which is constitutive to the yeast strain under the cultivation conditions reported in this work. The available evidence suggests that dye structure affects decolourisation rates but the basis for establishing a correlation between those two parameters remains, so far, unknown.

#### Acknowledgments

This work was partially funded by JNICT/Praxis XXI/2/2.2/QUI/44/94 (Portugal) and by the University of Minho. H.S. was a student from the Graz University of Technology (Austria) doing an individual project at the Chemistry Department, University of Minho, supported by the Erasmus programme.

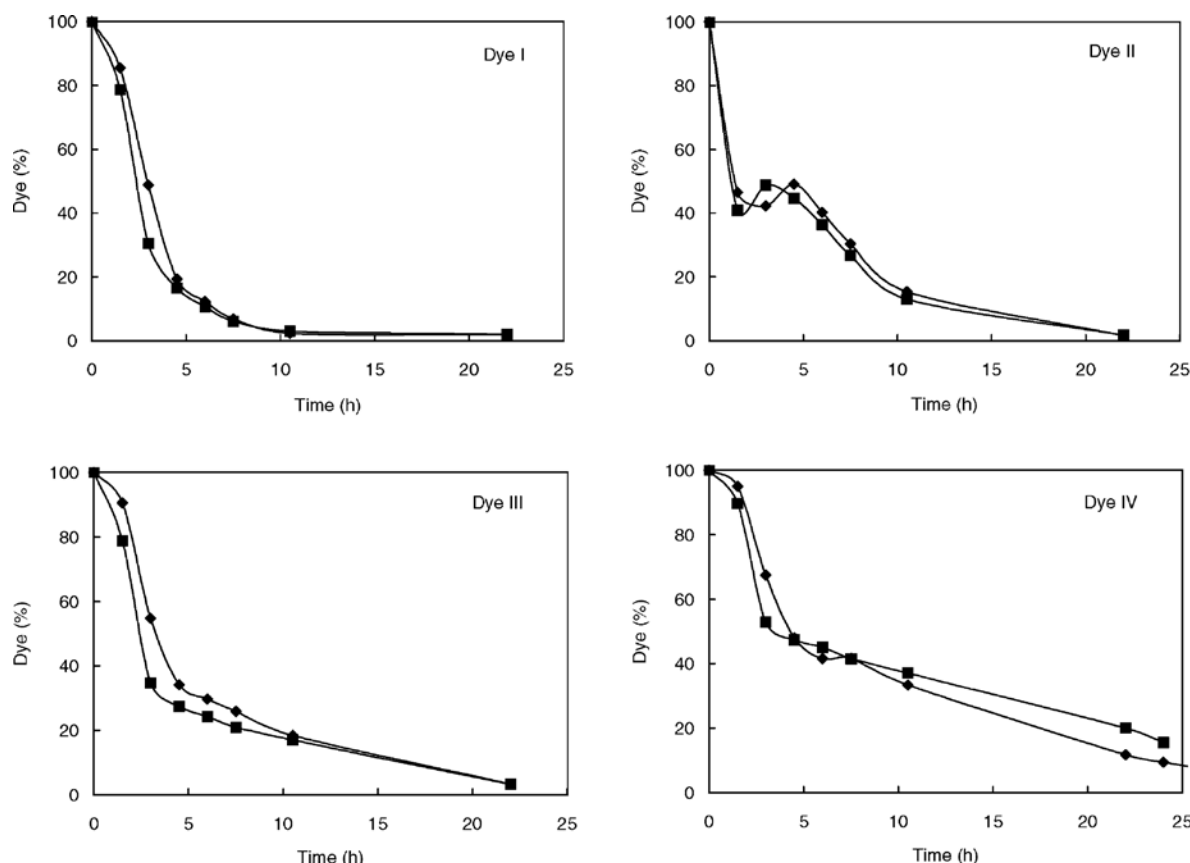


Fig. 5. Time-course of the decolourisation of Dyes I-IV by pre-adapted (■) and non-adapted (◆) cells of *C. zeylanoides* (for details, see text).

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## **CHAPTER 3**

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### **AZO REDUCTASE ACTIVITY IN *ISSATCHENKIA* *OCCIDENTALIS***

# Characterization of Azo Reduction Activity in a Novel Ascomycete Yeast Strain

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## Abstract

Several model azo dyes are reductively cleaved by growing cultures of an ascomycete yeast species, *Issatchenkia occidentalis*. In liquid media containing 0.2 mM dye and 2% glucose in a mineral salts base, more than 80% of the dyes are removed in 15 h, essentially under microaerophilic conditions. Under anoxic conditions, decolorization does not occur, even in the presence of pregrown cells. Kinetic assays of azo reduction activities in quasi-resting cells demonstrated the following: (i) while the optimum pH depends on dye structure, the optimum pH range was observed in the acidic range; (ii) the maximum decolorizing activity occurs in the late exponential phase; and (iii) the temperature profile approaches the typical bell-shaped curve. These results indirectly suggest the involvement of an enzyme activity in azo dye reduction. The decolorizing activity of *I. occidentalis* is still observed, although at a lower level, when the cells switch to aerobic respiration at the expense of ethanol after glucose exhaustion in the culture medium. Decolorization ceased when all the ethanol was consumed; this observation, along with other lines of evidence, suggests that azo dye reduction depends on cell growth. Anthraquinone-2-sulfonate, a redox mediator, enhances the reduction rates of the *N,N*-dimethylaniline-based dyes and reduces those of the 2-naphthol-based dyes, an effect which seems to be compatible with a thermodynamic factor. The dye reduction products were tested as carbon and nitrogen sources. 1-Amino-2-naphthol was used as a carbon and nitrogen source, and *N,N*-dimethyl-*p*-phenylenediamine was used only as a nitrogen source. Sulfanilic and metanilic acids did not support growth either as a carbon or nitrogen source.

## 1. Introduction

Over the last two decades, considerable work has been done with the goal of using microorganisms as bioremediation agents in the treatment of wastewater containing textile dyes. These contaminants contribute a minor fraction to the usually high load of dissolved organic matter in textile effluents (35), but they are highly visible and must be removed in order to comply with the regulations concerning effluent discharge.

Azo aromatic dyes are the major group of textile dyestuffs. These structures can be reductively cleaved into colorless amines by several bacterial species (for reviews, see references 2, 9, 41, and 43); nevertheless, azo dye reduction occurring in the presence of living matter can be an abiotic process. An example of this is the reduction of acid orange 7 (called dye IV in the present study) and reactive red 2 in anaerobic sludge, where sulfide, produced by sulfate-reducing microorganisms, can reduce azo bonds (45). In most of the reported processes of azo dye bioreduction, however, the participation of an enzymatic activity is assumed. Since the products of azo dye reduction, with few exceptions (4, 11, 26), cannot be used by bacteria as carbon and energy sources, the cleavage of azo bonds is a gratuitous process which can occur when the microorganisms use a reduced carbon compound as the growth substrate.

Azo dye reduction occurring in the presence of living matter poses additional problems. Sulfonic azo dyes are impermeant to the cell membranes. NAD(P)H is also impermeant to the cell membranes and is believed to be the primary source of electrons. This is why reduction of highly polar azo dyes is usually postulated to take place outside the cell (43). This fact would be compatible either with an abiotic reduction mediated by some extracellular reductant species (45) or with the involvement of an externally directed reductase activity in the plasma membrane that was capable of transferring reducing equivalents to acceptor species in the extracellular medium. So far, several bacterial cytoplasmic azoreductases have been isolated and characterized (18, 33, 37, 38). However, as shown by two recent studies (5, 42), the putative azoreductases had, in fact, insignificant *in vivo* activity.

Independently of the intracellular location of the azoreductase, theoretically, a redox mediator could facilitate the transfer of reducing equivalents from intracellular NAD(P)H to the substrate dye. This hypothesis was proposed as early as 1975 in a study describing the decolorization of azo food dyes by *Proteus vulgaris* (15). The participation of redox mediators in the decolorization of azo dyes by bacterial species is now generally accepted, and several mediators (usually quinonoid compounds) have been described as effectively enhancing decolorization processes (7, 22, 23, 40). The redox mediators studied are usually exogenous compounds added to the culture medium, but in a recent study (22), Keck and co-workers observed that aerobic preincubation of a *Sphingomonas* sp. strain with 2-naphthalenesulfonate strongly stimulated the subsequent anaerobic reduction of azo dyes by the same

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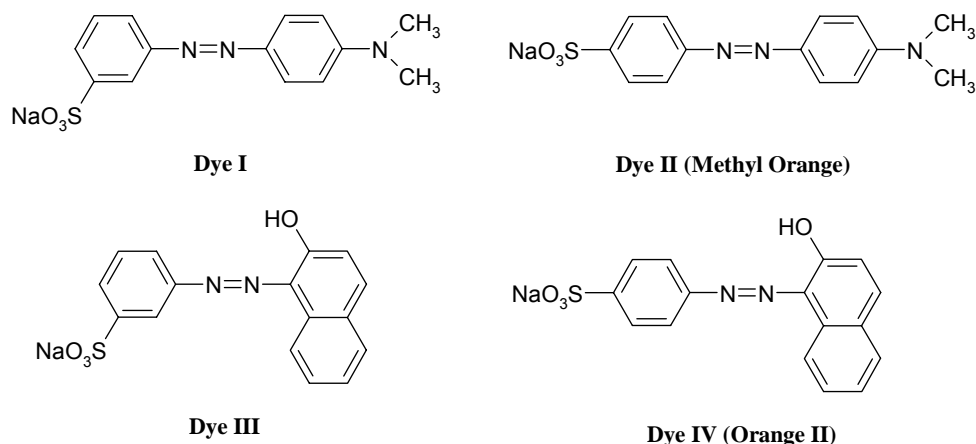


FIG. 1. Structures of the azo dyes used in this work: *m*-(4-dimethylamino)phenylazo]benzenesulfonic acid (dye I), *p*-(4-dimethylamino)phenylazo] benzenesulfonic acid (CI 13025 methyl orange or orange 52) (dye II), *m*-(2-hydroxy-1-naphthyl)azo]benzenesulfonic acid (dye III), and *p*-(2-hydroxy-1-naphthyl) azo]benzenesulfonic acid (CI 15510 orange II or acid orange 7) (dye IV).

strain. In a later study (23), Keck et al. identified 4-amino-1,2-naphthoquinone and 4-ethanolamino-1,2-naphthoquinone, formed by spontaneous oxidation of 1,2-dihydroxynaphthalene (an intermediate of the 2-naphthalenesulfonate degradation), as putative redox mediators in the decolorization reaction. Another study, observing an autocatalytic effect in the anaerobic reduction of acid orange 7, demonstrated that 1-amino-2-naphthol, one of the dye reduction products, stimulated decolorization (46). The presence of redox mediators in humic substances naturally found in soils has also been reported to enhance decolorization and other redox processes (10, 16, 23).

Azo dye reduction occurs preferentially under anoxic or oxygen-limited conditions (6, 20, 24, 28, 32, 34, 40, 48). Under these circumstances, azo dyes act as terminal electron acceptors during microbial respiration. Aerobic azo dye reduction processes in the presence of additional carbon sources were recently questioned by Stolz (43), since the experimental conditions of decolorization have not unequivocally shown the presence of oxygen in the culture medium, being more consistent, in fact, with oxygen-limited conditions.

The few reports on bioremediation of colored effluents by yeasts usually mention biosorption as the major cause for decolorization (14, 31). Martins et al., however, isolated a strain of *Candida zeylanoides*, which efficiently decolorizes several azo dyes (30). Further work demonstrated that a reductive cleavage of the azo bond was involved in this process and described some characteristics of the corresponding dye reducing activity (39). In this work, we examined the decolorizing activity of a novel yeast strain, *Issatchenkia occidentalis*. This species is even more efficient than *C. zeylanoides* in decolorizing the previously tested dyes. Therefore, we decided to investigate, in more detail, the effects of several parameters on the performance of *I. occidentalis* as an azo dye reducer. The evidence obtained from this study is expected to provide a sound basis for the

development of a biotreatment process for azo dye-containing wastewaters.

#### MATERIALS AND METHODS

**Chemicals and culture medium components.** Peptone, yeast extract, yeast nitrogen base (YNB), and yeast carbon base (YCB) were obtained from Difco. Other chemicals were commercially available, analytical grade reagents.

**Dyes.** The structures of the dyes tested in the present work are depicted in Fig. 1. Dye II (methyl orange, CI 13025) and dye IV (orange II, CI 15510), both ca. 85% dye content, were purchased from Sigma-Aldrich and used without further purification. The other two dyes (minimum 90% dye content) were synthesized by the conventional method of coupling the diazonium salt of metanilic acid with either *N,N*-dimethyl-*p*-phenylenediamine or 1-amino-2-naphthol (17). The structures of the isolated dyes, as sodium salts, were confirmed by <sup>1</sup>H nuclear magnetic resonance spectroscopy in dimethyl sulfoxide.

**Microorganism and maintenance conditions.** The ascomycete yeast *I. occidentalis* (PYCC 5770) (deposited in the Portuguese Yeast Culture Collection) was isolated on the basis of its capacity to decolorize YEPD (yeast extract-peptone-dextrose) agar plates containing 0.5% [wt/vol] yeast extract, 1% [wt/vol] peptone, 2% [wt/vol] glucose and the azo dye orange II, as described in a previous publication (30). The identification of the yeast strain followed the usual methods described for yeast taxonomy (44): testing of defined carbon compounds as fermentation substrates or as the sole carbon and energy sources, assimilation tests of nitrogen compounds, temperature tolerance for growth (30, 35, and 40°C), growth in the presence of 0.01 and 0.1% (wt/vol) cycloheximide, growth in the presence of 50 and 60% (wt/vol) D-glucose, and hydrolysis of urea. Morphological characteristics were also considered, i.e., vegetative reproduction only by budding, formation of pseudohyphae, and formation of persistent asci containing round ascospores on yeast malt agar. A commercial software (3) was used as an aid for species identification. The strain was routinely maintained on slants of YEPD agar.

**Analytical methods.** Cell growth in liquid medium was monitored by attenuation measurements (47) at 640 nm ( $D_{640}$ ). Blanks for these readings were prepared from aliquots of centrifuged medium. At high cell densities, both sample and blank were diluted with distilled water by the same factor. A linear correlation between attenuation readings ( $D_{640}$ ) and cell weight (dry weight) was established by the standard gravimetric method (cell weight [dry weight] in grams.liter<sup>-1</sup> = 1.1289( $D_{640}$  - 0.0487);  $r^2$  = 0.996). Dye concentration was monitored by absorbance readings of aliquots of centrifuged medium at the dye  $\lambda_{max}$ . The assay cuvette contained 0.3 ml of 1 M acetate buffer (pH 4.0), sample, and water to a volume of 3.0 ml; the blank was prepared with the same dilution of buffer in distilled water. Dissolved oxygen was measured as partial oxygen pressure using a Clark-type polarographic electrode and an ATI RUSSEL model RL 400 instrument according to the

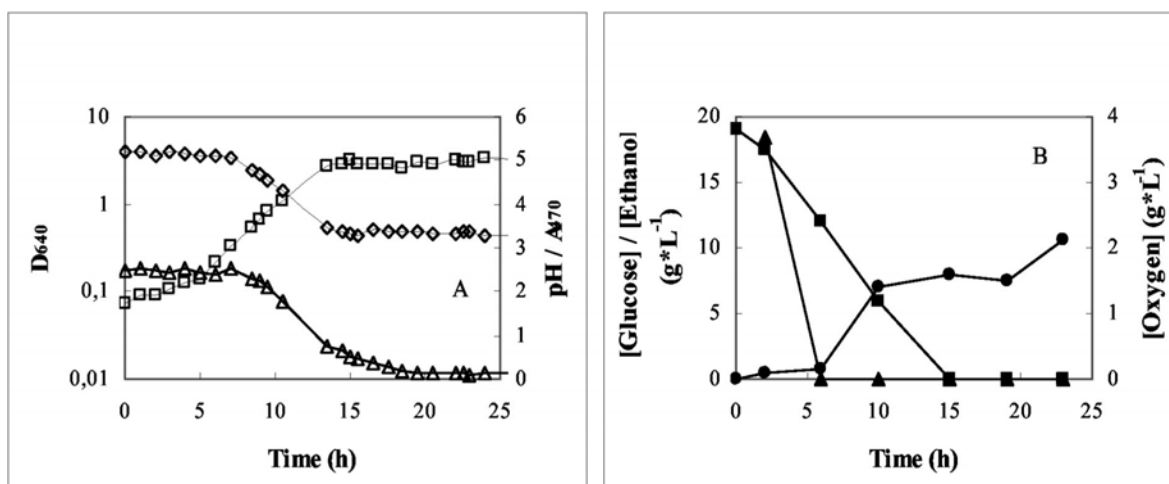


FIG. 2. Typical growth (□), pH (◇), and decolorization curves (Δ) (A) and concentrations of glucose (■), ethanol (●) and oxygen (▲) (B) during incubation of *I. occidentalis* on NDM containing 0.2 mM dye II. The initial cell density ( $D_{640}$ ) was 0.09.

manufacturer's instructions (detection level, 0.1  $mg \cdot liter^{-1}$ ). Metanilic and sulfanilic acids, formed by reduction of dye I or III and dye II or IV, respectively, were detected and quantified by high-pressure liquid chromatography (HPLC) analysis, using a reversed-phase column (RP-18) and an eluent containing the ion-pair reagent tetrabutylammonium phosphate (TBAP), as previously described (39). Under these conditions, metanilic and sulfanilic acids had retention times of  $10.0 \pm 0.5$  and  $12.4 \pm 0.5$  min, respectively. Glucose and ethanol concentrations in the growth media were also determined by HPLC (retention times of  $11.1 \pm 0.2$  and  $24.5 \pm 0.2$  min, respectively), using a Polyspher OAKC (Merck) column, refractive index detection, and arabinose as the internal standard (27).

**Decolorization in liquid media.** Decolorization experiments by growing cultures of *I. occidentalis* were typically performed in 250-ml cotton-plugged Erlenmeyer flasks with 100 ml of sterile medium (normal decolorization medium [NDM]) containing 2% glucose as a carbon and energy source and the test dye (0.2  $mmol \cdot liter^{-1}$ ) in a mineral salts base, as previously described (39). The cultures were inoculated at a low cell density with a cell suspension prepared from a freshly grown slant. The flasks were incubated under orbital shaking (120 rpm) at 26°C, except where stated otherwise. Biomass and concentrations of dye, sulfanilic or metanilic acid, glucose, and ethanol were measured as described above, in samples of the incubation mixture collected at defined times. Dissolved oxygen was measured directly in the incubation media. When testing the effect of adaptation of the strain to the dyes on the decolorization process, the cells were pregrown in NDM with or without dye, until the mid-exponential growth phase was reached. The cells were then harvested by centrifugation at 5,000 rpm in an Eppendorf Centrifuge 584OR, washed twice in cold sterile distilled water, and used to inoculate fresh sterile NDM containing 0.2  $mmol$  of dye  $\cdot liter^{-1}$ . The cultures were started at high cell densities (1.4  $D$  units; 1.6 g [dry wt] of cells  $\cdot liter^{-1}$ ). In control experiments, the inocula consisted of unadapted cells.

**Assimilation of azo dye reduction products.** Minimal media with all essential nutrients and vitamins except a carbon source (YNB) or a nitrogen source (YCB) were prepared according to the manufacturer's instructions and supplemented with 0.5 mM metanilic or sulfanilic acid. Appropriate volumes of these media (100 ml) in 250-ml Erlenmeyer flasks were inoculated with freshly grown cells and incubated at 26°C and 120 rpm. Cell growth was monitored as described above. Control experiments were performed with 0.5 mM ammonium sulfate as the sole nitrogen source in YCB or with 0.5 mM glucose as the sole carbon and energy source in YNB. Using the same method, 1-amino-2-naphthol and *N,N*-dimethyl-*p*-phenylenediamine, both 0.5 mM, were tested as nitrogen sources. The initial pH was adjusted to  $5.2 \pm 0.2$ .

**Activity assays with resting cells.** The decolorization activities of nongrowing cells were determined at various pH values, with cells harvested in different growth stages and with or without redox by using the redox mediator anthraquinone-2-sulfonic acid (AQS). To investigate the effect of pH or temperature on decolorization activity, the cells were grown for ca. 11 h on NDM, harvested by centrifugation, washed, and resuspended in 0.05 M phosphate buffer solution of the required pH. The assay mixture contained, in a total volume of 20 ml, 10.4 mM glucose and 0.047 mM dye; 0.1 mM AQS was included in activity assays of dyes I and II and omitted in the assays of dyes III and IV. The assay mixtures

typically contained  $7.1 \pm 0.3$  g of cells (dry wt)  $\cdot liter^{-1}$ . The assays were performed in 2 h in 50-ml cotton-plugged Erlenmeyer flasks shaken at 120 rpm and at 26°C. Within this period, the dye concentration decreased linearly with time. The same procedure was followed with cells harvested in different growth phases.

## RESULTS

**Dye removal by growing cultures.** In decolorization assays performed with dyes I to IV, the exponential growth phase was observed between 5 and 12 h of incubation, following a lag phase of ca. 4 h. The fastest decrease in dye concentration occurred during the late exponential growth phase. After 20 h of incubation, the percentages of dye removal were >95% for dyes I and II and ca. 85% for dyes III and IV. Figure 2A shows a typical growth curve, as well as the evolution of the medium pH and dye II concentration versus time, whereas Fig. 2B displays the variation in glucose, ethanol, and dissolved oxygen concentrations within the same period.

HPLC analysis of supernatant samples for the presence of dye reduction products revealed the presence of metanilic acid or sulfanilic acid during decolorization of dyes I (or III) and II (or IV), respectively. These acids are stable under the experimental conditions, and as shown below, they are not used by this yeast as a carbon and energy source or as a nitrogen source within the experiment period. The maximum concentrations of these sulfonates in decolorized media approached the stoichiometric levels ( $0.17 \pm 0.02$  mM).

Ethanol starts to accumulate in the culture medium at the onset of the exponential growth phase, indicating that *I. occidentalis* is a fermentative yeast. Since the maximum yield of ethanol is 0.51 g  $\cdot$  g of glucose $^{-1}$ , the observed concentration of ethanol in the culture medium (Fig. 2B) approaches the expected value.

**Effects of oxygen.** In the standard decolorization experiments described above, the incubation mixtures were contained in cotton-plugged Erlenmeyer flasks and subjected to agitation (120 rpm). Under these conditions,



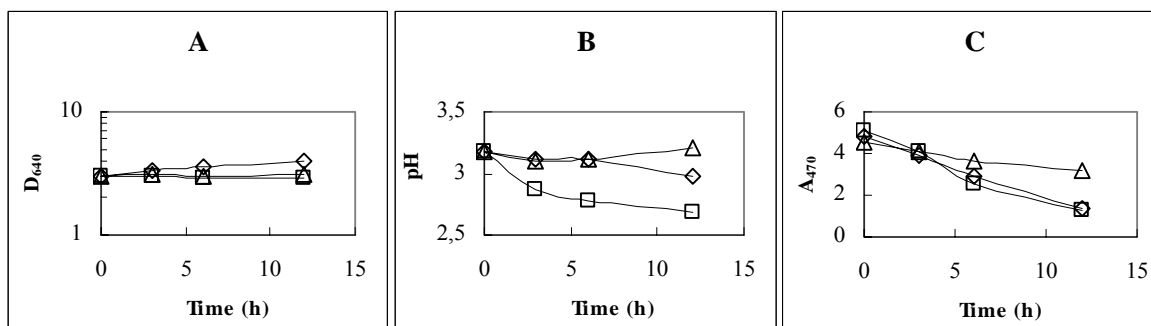


FIG. 3. Variation of cell density measured as  $D_{640}$  (A), pH (B), and dye concentration measured as  $A_{470}$  (C) in NDM preadjusted to pH 3.2 containing 0.2 mM dye II under different conditions: anoxic conditions ( $\Delta$ ), air-flushed cultures ( $\square$ ), and normal incubation at 120 rpm ( $\diamond$ ) (see text for details). The initial cell density was  $\approx 3$  D units.

oxygen (from air) is admitted in the system, but  $\text{CO}_2$  evolution, resulting from glucose fermentation, contributes to decrease its concentration in the medium. In fact, analysis of Fig. 2B shows that, after 6 h of incubation, dissolved oxygen concentration is  $0.2 \text{ mg} \cdot \text{liter}^{-1}$  or lower. Decolorization, which is faster during the late exponential phase, i.e., between 8 and 11 h of incubation (Fig. 2A), therefore occurs under low oxygen concentrations.

In an attempt to further elucidate the effect of dissolved oxygen in the decolorization process, additional decolorization experiments with cell suspensions inoculated at high densities (ca. 3.0 D units) were performed. Cells were grown for 10 h under the usual conditions and then harvested by centrifugation and washed. The combined pellets were resuspended in fresh NDM with 0.2 mM dye II, and 100-ml volumes of the resulting suspension were placed in separate Erlenmeyer flasks. The aeration conditions were as follows: one flask was continuously flushed with sterile air and incubated while being stirred magnetically, and a second flask was flushed with nitrogen for 15 min, tightly plugged with a rubber stopper, and placed in the orbital incubator. A control experiment, in our standard conditions, was simultaneously run. In the subsequent 12 h of incubation, a very slight increase in growth was detected (Fig. 3A), but in the flasks incubated under the standard conditions or with oxygen flushing, a slight decrease in the pH was observed (Fig. 3B). This was taken as evidence of metabolic activity, which supported a decolorization of ca. 70%, in both cases (Fig. 3C). The dissolved oxygen concentrations were  $3.9 \pm 0.4 \text{ mg} \cdot \text{liter}^{-1}$  in the aerated culture and  $<0.2 \text{ mg} \cdot \text{liter}^{-1}$  in the control culture. Under anoxic conditions, the color loss was only ca. 20%, as seen in Fig. 3, and oxygen concentration remained below the detection level.

The absence of metabolic activity in the anoxic conditions, obtained as described above, was further confirmed through the observation that a culture started at a low cell density and incubated for 24 h failed to grow (results not shown).

**Effects of dye.** Possible toxic effects of xenobiotic compounds on a microorganism can be preliminarily assessed by comparing its growth curves in the presence and absence of the tested compounds. For dyes I to IV, it

has been observed that neither the specific growth rate ( $0.32 \pm 0.03 \text{ h}^{-1}$ ) nor the biomass yield ( $0.16 \pm 0.02 \text{ g} [\text{dry weight}] \cdot \text{g of glucose}^{-1}$ ) were significantly affected by the presence of the dyes.

Having excluded the possibility that the dyes themselves were toxic, we also tested a possible enhancement of the decolorization process by using cell suspensions which had been grown overnight in NDM containing 0.2 mM dye as preinocula. The volume of each preinoculum was 20% of the final volume of the incubation mixture. Preinocula grown in the absence of dye were used in control experiments. For all the dyes tested, the decolorization curves obtained with adapted and nonadapted cells did not present any significant differences (Fig. 4), as observed earlier with *C. zeylanoides* (39).

#### Standard dye reduction assay with resting cells.

The apparent rates of dye removal by growing yeast cells are affected by increasing biomass and, presumably, by pH. A study of the parameters affecting the kinetics of decolorization therefore requires buffered media and a fixed amount of cells. This condition was approached by establishing the glucose concentration that allowed the best compromise between minimum cell growth and measurable dye reduction activity. Glucose concentrations of up to 20 mM in 0.05 M phosphate

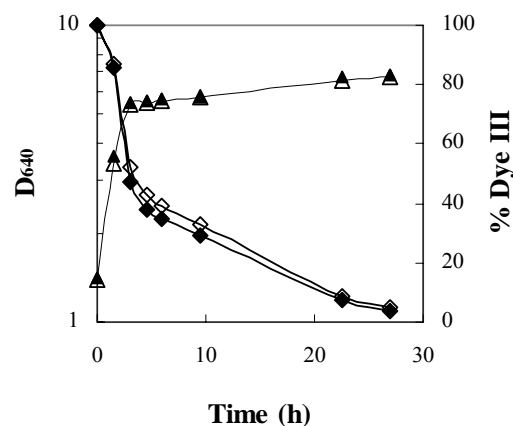


FIG. 4. Growth of *I. occidentalis* (triangles) and decolorization curves (diamonds) of dye III by preadapted cells (open symbols) and nonadapted cells (closed symbols) on NDM.

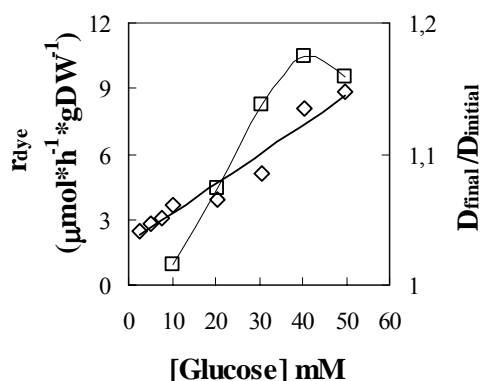


FIG. 5. Effect of glucose concentration on specific decolorization rates,  $r_{\text{dye}}$  ( $\diamond$ ) and cell growth (final  $D_{640}$ /initial  $D_{640}$  ratio) ( $\square$ ).  $r_{\text{dye}}$  is given in micromoles  $\cdot$  hour $^{-1}$   $\cdot$  gram of cells (dry wt [DW]) $^{-1}$ .

buffer (pH 4.0) produced a final  $D_{640}$ /initial  $D_{640}$  ratio of  $\leq 1.1$  in the course of 2 h, the maximum time for the assays (Fig. 5). For a glucose concentration of 10.4 mM in assay mixtures, as used henceforth, the final  $D_{640}$ /initial  $D_{640}$  ratio was  $< 1.05$  and the measured specific activities therefore approached those of resting cells.

The dye concentration used (0.047 mM) allowed direct absorbance readings, thus minimizing errors related to sample dilution. The optimum biomass concentration was determined by performing assays with various cell concentrations from 0 to ca. 14  $D$  units (0 to 15.8 g of cells [dry wt]  $\cdot$  liter $^{-1}$ ). The initial decolorization rates, as determined from plots of dye absorbance versus time, in each assay mixture, displayed saturation like kinetics, due to substrate limitation at higher biomass concentrations. In terms of specific decolorization rates, the optimum cell concentration ranged between 4 and 6  $D$  units (4.5 to 6.7 g of cells [dry wt]  $\cdot$  liter $^{-1}$ ), decreasing for  $D$  values above 6, as seen in Fig. 6A.

The effect of the redox mediator AQS on the specific decolorization rate of dye II was also analyzed. The assay mixtures contained optimum cell and glucose concentrations, as defined above, and AQS concentrations ranging from 0 to 0.445 mM. The decolorization rate increased linearly, reaching a maximum threefold increase with an AQS concentration of 0.1 mM. The effect of further concentration increase was negligible (Fig. 6B). For subsequent dye reduction assays with this dye, and also with dye I, which displayed the same type of response, a 0.1 mM concentration of AQS was therefore adopted. However, in decolorization assays of dyes III and IV, AQS was omitted because of its negative effect (results not shown).

**Kinetic assays.** The assays for assessing the effects of dye concentration, pH, temperature, and growth phase on the decolorization rate were performed under the optimized conditions of glucose concentration and cell density, as described above, with added AQS in assays with dyes I and II only. Except when studying the effect of pH, cells were suspended in 0.05 M phosphate buffer,

pH 4.0. The cells used in the assays had previously been grown for 10 to 11 h as described in Materials and Methods. However, in the assays investigating dye reduction activities in different growth phases, the cells were harvested at the specified times. The data from dye reduction assays performed with various concentrations of dye II as the substrate could be fitted adequately to the Michaelis-Menten equation,  $r_{\text{dye}} = r_{\text{dye,max}} [\text{dye}]/(K_m + [\text{dye}])$ , where  $r_{\text{dye}}$  and  $r_{\text{dye,max}}$  are the initial and maximum decolorization rates, respectively,  $K_m$  is the Michaelis constant (millimolar) and  $[\text{dye}]$  is the concentration of the dye (millimolar). The kinetic parameters obtained with the Hanes linearization of the experimental data were as follows:

$r_{\text{dye,max}} = 3.2 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$  and  $K_m = 0.034 \text{ mM}$  ( $r^2 = 0.995$ ).

The effect of pH on the decolorization rate was studied by using cell suspensions in phosphate buffers of pH ranging from 3 to 7. Typically, for dyes I and II, the decolorization rate was virtually constant between pH values of 3 to 5, showing a fast decrease in the range 6 to 7. In contrast, for dyes III and IV, the decolorization rates decreased with pH increase in the range 3 to 6. Within the pH range tested, the maximum decolorization rate was observed, in both cases, at pH 3. Figure 7A illustrates this behavior for dyes II and III.

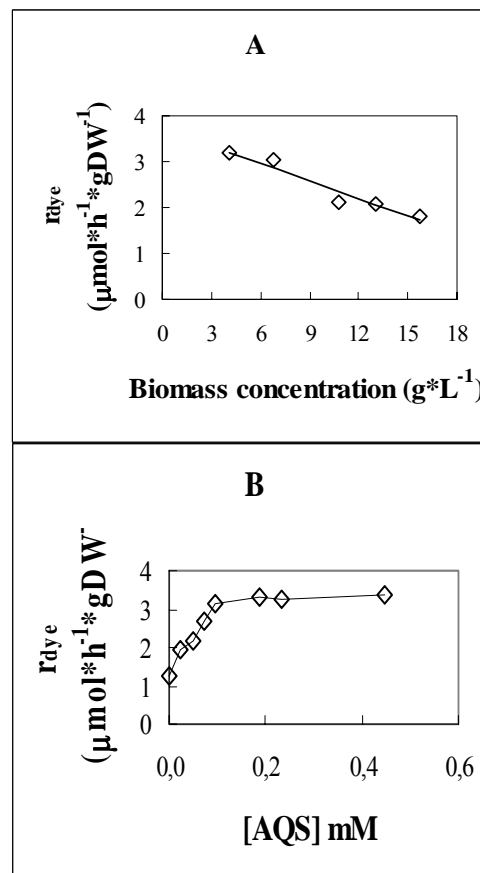


FIG. 6. Effects of biomass (A) and AQS concentration (B) on specific decolorization rates ( $r_{\text{dye}}$ ) of dye II.  $r_{\text{dye}}$  is given in micromoles  $\cdot$  hour $^{-1}$   $\cdot$  gram of cells (dry wt [DW]) $^{-1}$ .

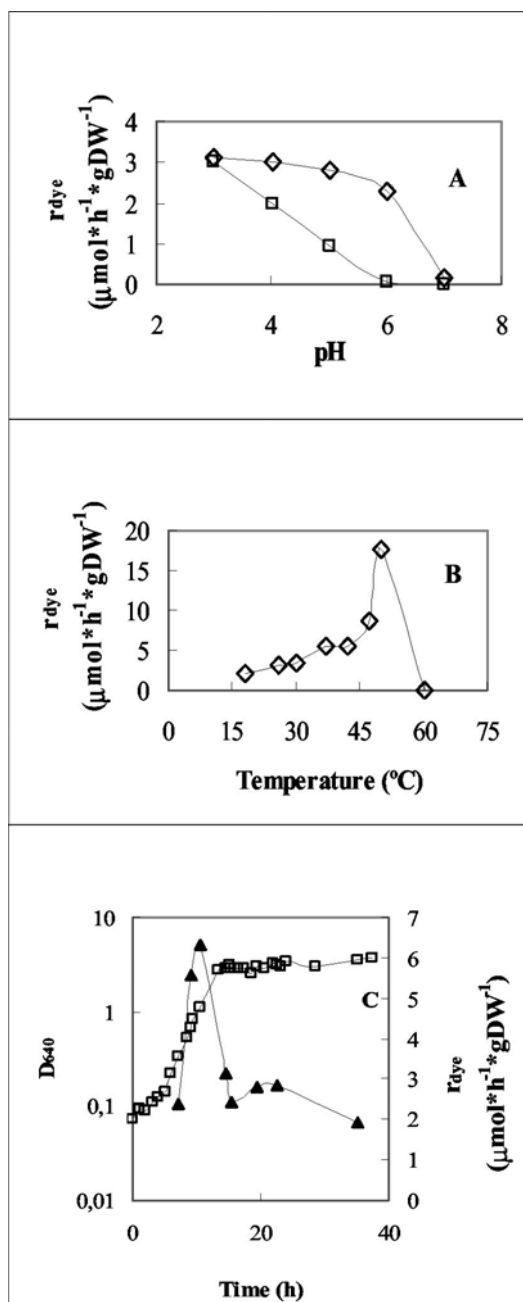


FIG. 7. Effects of pH (A), temperature (B), and growth phase (C) on specific decolorization rates ( $r_{dye}$ ) (see text for details). (A) Effects of pH on dye II ( $\diamond$ ) and dye III ( $\square$ )  $r_{dye}$ ; (B) effect of temperature on dye II ( $\diamond$ )  $r_{dye}$ ; (C) effect of growth phase on dye II ( $\square$ )  $r_{dye}$  and a typical growth curve ( $\blacktriangle$ ) for *I. occidentalis* in the presence of dye II.  $r_{dye}$  is given in micromoles  $\cdot$  hour $^{-1}$   $\cdot$  gram of cells (dry wt [DW]) $^{-1}$ .

The effect of temperature on the decolorization rate was tested in the 18 to 60  $^{\circ}\text{C}$  temperature range. As can be seen from Fig. 7B, the temperature profile of the decolorization reaction approaches the expected bell-shaped form characteristic of enzyme activity. The decolorization rates varied from 2.0 (at 18  $^{\circ}\text{C}$ ) to 17.7  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$  (at 50  $^{\circ}\text{C}$ ), indicating that within the assay

period (2 h) and at temperatures up to 50  $^{\circ}\text{C}$ , there was no apparent reduction in the decolorizing activity of the cells. Complete loss of activity was observed at 60  $^{\circ}\text{C}$ . Azo reduction assays were also performed with yeast cells harvested by centrifugation at different times, while growing in NDM. The cells were washed and then resuspended in the standard assay mixture. As seen in Fig. 7C, a sharp activity maximum is observed for cells in the late exponential phase, with the specific decolorization rate for dye II reaching the value 6.3  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$  at 10.5 h of incubation. During the early stationary phase, the rate remained approximately constant (2.8  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ ), decreasing to 1.9  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$  after 35 h.

**Assimilation of dye reduction products.** The persistence of metanilic or sulfanilic acids in decolorized supernatants of cultures incubated for a long period suggested that none of these compounds could be assimilated by the yeast strain (results not shown). This was confirmed by incubation of *I. occidentalis* in minimal medium (YCB or YNB) supplemented with a 0.5 mM concentration of the appropriate source. As seen in Table 1, none of the aminobenzenesulfonates supported yeast growth as a source of carbon and energy or nitrogen. When N,N-dimethyl-*p*-phenylenediamine was added to YCB minimal medium as a nitrogen source, yeast growth was observed. The other amine, 1-amino-2-naphthol, was used as a source of carbon and energy and of nitrogen by this yeast. Whenever assimilation occurred, an acidification of the medium was observed (final pH of  $3.5 \pm 0.5$ ).

**Sequential batch decolorization of dye II.** Standard decolorization experiments were usually monitored over a period up to 24 h. By this time, glucose was exhausted from the medium and dyes I and II had been completely removed; for dyes III and IV, a residual amount of 15 to 20% was still present. To examine the decolorization activity of the yeast cells after glucose depletion, a decolorized culture (300 ml), which had been incubated for 24 h under the standard conditions, was subjected to five successive additions of the required amounts of dye II stock solution to restore the initial dye concentration. Each dye addition was done after complete decolorization of the medium. As seen in Fig. 8, decolorization was complete during the first five cycles. During this process, ethanol concentration first increased to a maximum of 10 g  $\cdot$  liter $^{-1}$  in the course of the first cycle and then gradually decreased, disappearing at 160 h of incubation. By this time, decolorization also ceased. In the course of successive cycles, the concentration of sulfanilic acid gradually increased, as expected. Cell growth also continued, with an increase from 2.7 to 6.1  $D_{640}$  units, showing that the yeast switched from a fermentative metabolism, with glucose, to aerobic respiration, with ethanol. The dissolved oxygen concentration consistently remained below the detection level until the end of the experiment. The decolorizing activity of the yeast cells, measured by the standard assay, slowly decreased with the decolorization-dye addition cycles but was still present in the last sample of cells, collected after 200 h of incubation.

**TABLE 1.** Biomass yields of *I. occidentalis* on sulfanilic and metanilic acids and 1-amino-2-naphtol and N,N-dimethyl-p-phenylenediamine as carbon or nitrogen sources.

Minimal medium	Biomass yield, g.mmol <sup>-1</sup> (lag phase, h)					
	Glucose	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Sulfanilic Acid	Metanilic Acid	1-amino-2-naphtol	N,N-dimethyl-p-phenylene diamine
YCB		2.77 (-)	NG	NG	0.89 (12)	0.89 (2)
YNB	0.09 (2)		NG	NG	0.43 (-)	NG

NG, no growth

## DISCUSSION

Decolorization of azo dyes by yeasts is much less studied than the homologous process mediated by bacterial species. In this work, an attempt was made to elucidate some basic physiologic aspects associated with azo dye destruction by *I. occidentalis*. This yeast, like a previously described ascomycete yeast strain (39), is capable of reducing several monoazo dyes to the corresponding amines and, therefore, promotes their decolorization through the cleavage of the azo bond.

The decolorizing activity of *I. occidentalis* was first studied in actively growing cultures in a glucose-containing culture medium with moderate shaking (Fig. 2). Under these conditions, ethanol was detected in the culture supernatant, showing that the microorganism was fermenting glucose.

The similarities of the decolorization profiles by dye-adapted and nonadapted cells (Fig. 4) indicate that the

azo dye reduction activity in yeast cultures is a constitutive property of the cells, independent of their previous exposure to the dye. So far, we have not been able to detect any decolorizing activity in cell extracts or in the supernatant broth, even when high [NADH]/[dye] ratios were used. It thus appears that azo dye reduction activities are dependent on intact, active cells. Thus far, even in bacteria, the major enzyme responsible for azoreductase activity *in vivo* has not been positively identified. A recent report (42) described a high *in vitro* azoreductase activity of a cytoplasmic flavin reductase, part of the ribonucleotide reductase complex in *Escherichia coli*, but its overexpression in a *Sphingomonas* sp. strain failed to significantly increase the *in vivo* reducing activity of the bacterium. Earlier, Kudlich and coworkers (24) had also detected an azoreductase activity in a membrane fraction of the same *Sphingomonas* sp. strain. According to these researchers, the NADH:ubiquinone oxidoreductase was a likely

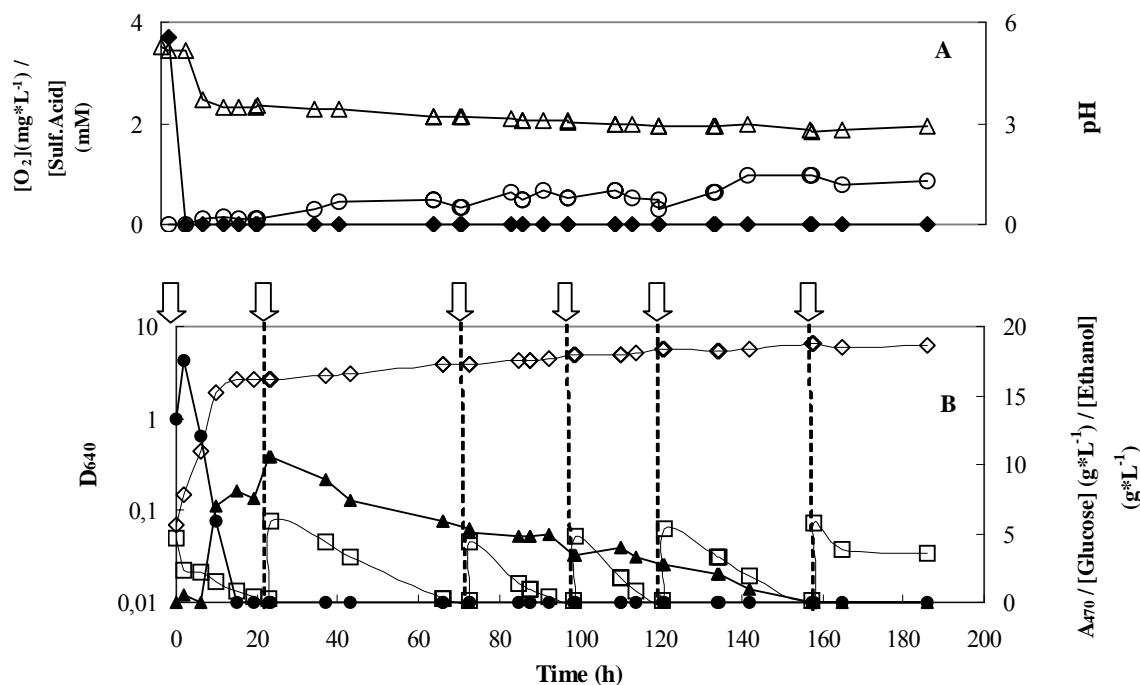


FIG. 8. Monitoring of several parameters during successive cycles of addition of dye to a culture of *I. occidentalis* on NDM, incubated at 120 rpm, without any further nutrient addition. Wide white arrows indicate the times of dye addition (see text for details). (A) pH ( $\Delta$ ) and concentrations of oxygen ( $\blacklozenge$ ) and sulfanilic acid [Sulf.Ac] ( $\circ$ ); (B) cell growth ( $\diamond$ ) and concentrations of glucose ( $\bullet$ ), ethanol ( $\blacktriangle$ ), and dye II ( $\square$ ).

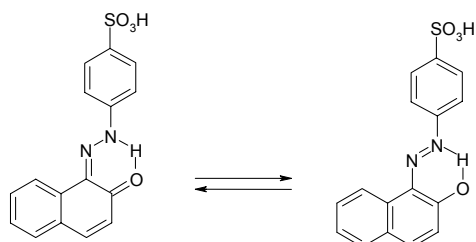
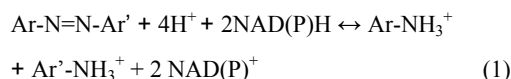


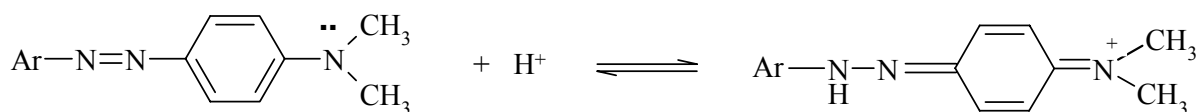
FIG. 9. Azo-hydrazone tautomers of dye IV.

candidate for the azoreductase activity. Dissolved oxygen is repeatedly considered an inhibitor of the dye bioreduction process (36), since both molecules act as electron acceptors and oxygen is a much stronger oxidant. This is, apparently, the reason why azo dyes are more readily reduced under anaerobiosis. The effect of oxygen was particularly addressed in this work with *I. occidentalis*, because in the preliminary experiments of the decolorizing potential of the yeast strains that we have studied so far, no attempt was made to prevent the access of oxygen to the dye-containing incubation medium. The decolorizing experiments were usually performed in cotton-plugged flasks, shaken at 120 rpm. Under these standard conditions, faster decolorization rates were indeed observed at low oxygen concentrations ( $<0.2 \text{ mg} \cdot \text{liter}^{-1}$ ), which can be considered microaerophilic (21). However, our results indicate that oxygen does not significantly interfere with color loss, as seen in air-flushed cultures (Fig. 3). A likely explanation for this fact is the high kinetic barrier involved in the reduction of the triplet ground-state dioxygen. In contrast, under anoxic conditions, i.e., in a nitrogen flushed culture incubated in rubber-stoppered flasks, the extent of decolorization was rather low (ca. 20%). Since pH (as well as cell density) essentially remained constant in the course of the experiment, a lack of metabolic activity was suspected. In a separate experiment that was also conducted under anoxic conditions in which the culture was inoculated at a low cell density, the yeast failed to grow. These observations show that *I. occidentalis* has an absolute requirement for oxygen, i.e., places it in the category of the aerobic-fermenting yeasts (1). Therefore, even pregrown cells were unable of performing azo dye reduction in the absence of oxygen. As far as we know, there is no previous report of a similar situation in decolorization processes mediated by facultative anaerobic bacteria. The effects of pH, temperature, dye and redox mediator concentrations, and growth phase on decolorization rates suggest the participation (direct or indirect) of an enzyme activity in azo dye reduction, because (i) the temperature profile of the azo reduction activity cannot easily be interpreted on the grounds of a purely abiotic process and (ii) the

decline of activity at the onset of the stationary phase is more compatible with a reduced metabolic activity of the cells. Additionally, azo dye reduction rate, like the intracellular formation of NAD(P)H, is a growth-dependent process, since it does not occur without a carbon and energy source. In fact, decolorization of dyes III and IV ceased upon glucose exhaustion in the culture medium, and decolorization of dye II, after the diauxic shift (Fig. 8), was linked to the presence of ethanol. The assays involving a redox mediator, AQS, produced opposing effects in the decolorization rates of the *N,N*-dimethylaniline-based dyes and 2-naphthol-based dyes: reduction rates increased for dyes I and II but decreased for dyes III and IV in the presence of AQS. The explanation for these apparently contradictory findings is likely to be purely thermodynamic, depending on the relative reduction potentials of dyes and mediator. The role of redox mediators in the process of azo dye reduction by yeasts therefore requires further investigation, but its effective usefulness in this yeast process is doubtful considering that only high [AQS]/[dye] ratios ( $>20$ ) produce significant rate enhancements. Concerning the effect of pH on the decolorization rates, there is also a considerable difference between *N,N*-dimethylaniline-based dyes (dyes I and II) and 2-naphthol-based dyes (dyes III and IV). Dyes with a hydroxyl group *ortho* to the azo bond, because they exist in the tautomeric azo-hydrazone forms (Fig. 9), have an abnormally high  $\text{pK}_{\text{a}2}$ ; for dye IV (and presumably for dye III),  $\text{pK}_{\text{a}2}$  is 11.4 (49). This means that the dye molecules will have an overall negative charge in the pH range 3 to 6. This fact alone is probably one factor making these dyes less susceptible to reduction than dyes I or II. Additionally, since the global reaction for dye reduction consumes four  $\text{H}^+$  ions from the medium plus the two hydrogen atoms from NAD(P)H (reaction 1), a decrease in  $[\text{H}^+]$  will have the effect of shifting the equilibrium in the direction of the reagents, thus making the free energy for the reaction less negative. The observed trend of an apparent dye reduction rate with increasing pH is consistent with the observation that the kinetics of redox reactions is usually determined by thermodynamics.



Dyes I and II, in contrast, have a  $\text{pK}_{\text{a}2}$  of  $\approx 3.5$ , and protonation occurs in an azo nitrogen (Fig. 10). This means that, up to  $\text{pH} \approx 5$ , a still considerable fraction of the molecules will be protonated in one azo nitrogen, apparently in an amount allowing a fast decolorization rate.

FIG. 10. Protonization of *N,N*-dimethylamino-based dyes at an azo nitrogen.

The above conclusions show that, in this respect also, the reduction by a yeast is considerably different from the reduction by bacteria, which has an optimum pH range close to neutrality (8, 33).

The decolorizing activity of *I. occidentalis* is not strictly related to a fermentative process, since it continued even after glucose exhaustion. Under these conditions, the yeast switches to aerobic respiration, at the expense of ethanol, which is the major carbon and energy source in the medium. Ethanol depletion, at 160 h of incubation, coincided with the disappearance of the decolorizing activity. Because the shake flasks were moderately agitated (120 rpm), the concentration of dissolved oxygen remained below the detection level. Therefore, the ethanol-dependent azo dye reduction equally occurred under microaerophilic conditions, although at a lower rate. The assimilation assays with sulfanilic and metanilic acids revealed their inability to be used as carbon and energy sources and also as nitrogen sources. This fact is not surprising, because except for a few works describing the bacterial degradation of sulfanilic acid (12, 13), 6-aminonaphthalene-2-sulfonate (19, 25), and 2-aminobenzenesulfonate (29), arenesulfonates are usually described as xenobiotic compounds. The persistence of the other two amines expected from dye reduction, N,N-dimethyl-*p*-phenylenediamine (dyes I and II) and 1-amino-2-naphthol (dyes III and IV), could not be detected by HPLC analysis, since they are quickly oxidized in aerated aqueous solutions, forming colored products, and their elution profiles change considerably within a short period of time. However, the colors associated with the oxidation products of either N,N-dimethyl-*p*-phenylenediamine (deep pink) or 1-amino-2-naphthol (dark yellow to brown) were not seen in decolorized media. The assimilation tests revealed that N,N-dimethyl-*p*-phenylenediamine was used as nitrogen source whereas 1-amino-2-naphthol was both a source of nitrogen and a source of carbon and energy, thus accounting for the absence of their oxidation products in the decolorized supernatants. This work shows that the ascomycete yeast *I. occidentalis* displays an effective azo dye reduction capacity, comparatively unspecific but, nevertheless, structure dependent in such aspects as glucose requirements for complete decolorization, pH-activity profiles, and effect of the tested redox mediator. All of these factors must be taken into account when considering the possible application of the yeast to a bioremediation process for azo textile dyes. Equally important is the effect of dissolved oxygen in the decolorization process. This particular strain, because it is an aerobic-fermentative yeast, has an absolute requirement of oxygen for growth and hence, for decolorization. While oxygen traces are sufficient for growth and allow dye reduction, our results also point to a noninhibitory effect of moderate levels of oxygen in the process. The decolorization process is also growth dependent, pointing to NAD(P)H availability as a crucial factor. This conclusion opens further perspectives in the search for more efficient yeast strains in azo dye decolorization.

## ACKNOWLEDGMENT

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## **CHAPTER 4**

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### **PREDICTING DYE BIODEGRADATION FROM REDOX POTENTIALS**



## Predicting Dye Biodegradation from Redox Potentials

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### Abstract

Two biological approaches for decolorization of azo sulfonated dyes have been compared: reductive decolorization with the ascomycete yeast *Issatchenkia occidentalis* and enzymatic oxidative decolorization with *Trametes villosa* laccase alone or in the presence of the mediator 1-hydroxybenzotriazole. The redox potential difference between the biological cofactor involved in the reductive activity of growing cells and the azo dye is a reliable indication for the decolorization ability of the biocatalyst. A linear relationship exists between the redox potential of the azo dyes and the decolorization efficiency of enzyme, enzyme/mediator, and yeast. The less positive the anodic peak of the dye, the more easily it is degraded oxidatively with laccase. The more positive the cathodic peak of the dye, the more rapidly the dye molecule is reduced with yeast.

### Introduction

Azo dyes are the largest group of synthetic dyes, applied to all fiber types. However, during the dyeing process not all of the dye is fixed on the textile material and up to 50% may be lost in the waste streams, depending on the dye type, depth of shade, and dyeing process. Conventional treatments of textile mill effluents do not remove efficiently most of these dyes (1). Biodegradability of the azo chromophores is based on oxidation and/or reduction reactions with enzymes, bacteria, or fungi (2). Under aerobic, anaerobic, or sequential anaerobic-aerobic treatments many bacteria reduce the azo bonds in dye molecules to aromatic amines, by the activity of low specificity azo-reductases (3-5). However, the azo dyes and their reductive biotransformation products (aromatic amines) have been shown to be toxic and in some cases mutagenic (6). Therefore, accumulation of these compounds should be avoided by using alternative dye decolorization approaches.

Laccase (EC 1.10.3.2) is a multicopper oxidase, which reduces oxygen to water and simultaneously performs one-electron oxidation of many aromatic substrates such as phenols and aromatic amines (7, 8). This enzyme decolorizes some azo dyes without direct cleavage of the azo bond through a highly nonspecific free radical mechanism, thereby avoiding the formation of toxic aromatic amines (9). However, the substrate specificity of laccase limits the number

problem laccase/mediator systems are normally used to broaden the range of azo dyes and to increase the decolorization rates (10-12).

The ability of the bioagents to degrade azo dyes depends on the structural characteristics of the dye, the temperature and pH of treatment, the presence of intermediates, and the difference between the redox potentials of the biocatalyst and the dye. This study discusses the biodegradation under aerobic conditions of azo dyes with yeasts with reducing activity and an oxidative enzyme laccase with or without mediator. These two approaches have been compared on the basis of the electrochemical properties of dyes and bioagents (13-15). The question targeted by this paper is whether the redox potential is a preliminary tool to predict the decolorization capacity of oxidative and reductive biocatalysts.

### Materials and Methods

**Dyes and Reagents.** The structures of the dyes and the mediator tested in the present work are depicted in Chart 1. Dyes I and III (minimum 90% dye content) were synthesized by the conventional method of coupling the diazonium salt of methanilic acid with either *N,N*-dimethyl-*p*-phenylenediamine or 1-amino-2-naphthol (16). The structures of the isolated dyes, as sodium salts, were confirmed by <sup>1</sup>H NMR spectroscopy in dimethylsulfoxide (DMSO). All other reagents and dyes were purchased from Sigma-Aldrich and used without further purification.

**Enzyme.** Laccase (EC 1.10.3.2) from *Trametes villosa* (5.3 mg protein/mL, 600 U/mL, supplied from Novo Nordisk, Denmark) was used for dye

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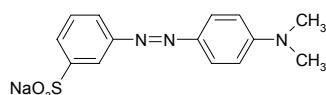
<sup>†</sup> Department of Textile Engineering, University of Minho.

<sup>‡</sup> Department of Biology, University of Minho.

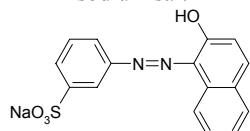
<sup>§</sup> Department of Chemistry, University of Minho.

<sup>|</sup> Graz University of Technology.

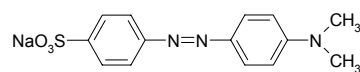
Chart 1. Dye and Mediator Structures



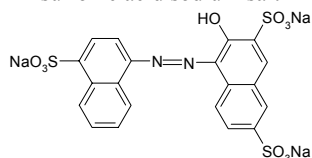
I) 3-(4-dimethylamino-phenylazo)-benzene sulfonic acid sodium salt



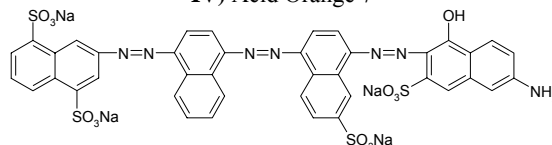
II) Acid Orange 52



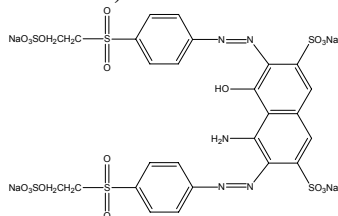
III) 3-(2-hydroxy-naphthalene-1-phenylazo)-benzene sulfonic acid sodium salt



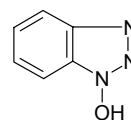
IV) Acid Orange 7



V) Acid Red 27



VI) Direct Blue 71



VII) Reactive Black 5

HBT – 1-HydroxyBenzoTriazole

decolorization at pH 5 (0.1 M Na acetate buffer) as previously described (17).

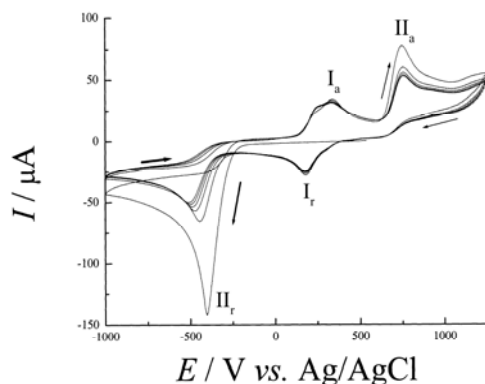
**Microorganism.** The ascomycete yeast *Issatchenkia occidentalis* (Portuguese Yeast Culture Collection 5770) was isolated on the basis of its capacity to decolorize agar plates containing yeast extract/peptone/glucose 0.5:1:2 (% w/v) and the azo dye Orange II, as described in a previous publication (18).

**Decolorization with Laccase and Laccase/Mediator System.** Dye solutions (0.1 mM; 2.5 mL) buffered with 0.1 M Na acetate buffer, pH 5, were incubated with 20  $\mu$ L of laccase (5.3 mg protein/mL, 600 U/mL) and 0.5 mL of distilled water in a standard stirred cuvette at 25°C. The dye absorbance was measured at different times during the experiment and the percentage of effluent decolorization thereof was calculated. In the case of experiments with mediator the water volume (0.5 mL) was replaced by 0.1 mM aqueous solution of 1-hydroxybenzotriazole (HBT).

**Dye Decolorization with Microorganism.** Decolorization experiments by growing cultures of *I. occidentalis* were typically performed in 250 mL cotton-plugged Erlenmeyer flasks with 100 mL of sterile medium (normal decolorization medium, NDM) containing 2% glucose, as carbon and energy source, and 0.2 mM tested dye, in a mineral salts base, as previously described (19). The flasks were

incubated under orbital shaking (120 rpm) at 26 °C. Dye concentration was monitored by absorbance readings of centrifuged medium aliquots at the dye  $\lambda_{\text{max}}$ . The assay cuvette contained 0.3 mL of 1 M acetate buffer (pH 4.0), sample, and water to 3.0 mL; the blank was prepared with the same dilution of buffer in distilled water.

**Electrochemical Measurements.** Cyclic voltammetry of the azo dyes was performed using a Voltalab 30 potentiostat (Radiometer Analytical, France), controlled by the Voltmaster 4 electrochemical software, at 100 mV s<sup>-1</sup> scan rate. The working, counter, and reference electrodes were, respectively, a glassy carbon electrode (0.07 cm<sup>2</sup>), coiled platinum wire (23 cm), and an Ag/AgCl electrode filled with 3 M NaCl, all purchased from BAS, USA. The glassy carbon electrode was successively polished with 5, 1, 0.3, and 0.05  $\mu$ m alumina polish (Buehler Ltd, USA) and then rinsed with 8 M nitric acid and distilled water before use. The experiments were performed in 0.1 M acetate buffer pH 5 at dye concentration of 0.1% w/v. Prior to analysis all solutions were purged with nitrogen for 15 min. The redox potentials recorded vs Ag/AgCl reference electrode were corrected by 0.206 V to the standard hydrogen electrode (SHE). Redox potentials of *T. villosa* laccase, 1-hydroxybenzotriazole, and nicotinamide adenine dinucleotide phosphate



**Figure 1.** Cyclic voltammogram of dye I: (thin line) positive to negative, (thick line) negative to positive; 6 cycles at 100 mVs<sup>-1</sup> scan rate.

(NADH) were provided from the literature and are as follows: laccase +780, HBT +1.084, and NADH -320 mV vs NHE (20-22).

## Results and Discussion

**Cyclic Voltammetry of Azo Dyes.** The azo dyes tested in this study presented similar cyclic voltammograms illustrated by the voltammogram of dye I (Figure 1), in both positive and negative scans. In the first positive scan of dye I an irreversible anodic peak (II<sub>a</sub>) in the potential range of +0.9 to +1.3 V vs NHE was observed. All dyes displayed an irreversible reduction peak in the range of -0.13 to -0.48 V vs NHE (II<sub>r</sub>). In the following scans an apparently semireversible redox couple (I<sub>a</sub>, I<sub>r</sub>) was detected. The reductive wave I<sub>r</sub> of the semireversible redox couple did not appear in the first negative scan. These redox couple peaks appear to be associated with the formation of unstable amine products, which were oxidized in the range of +0.15 to +0.58 V vs NHE and reduced in the potential range of -0.1 to +0.3 V vs NHE.

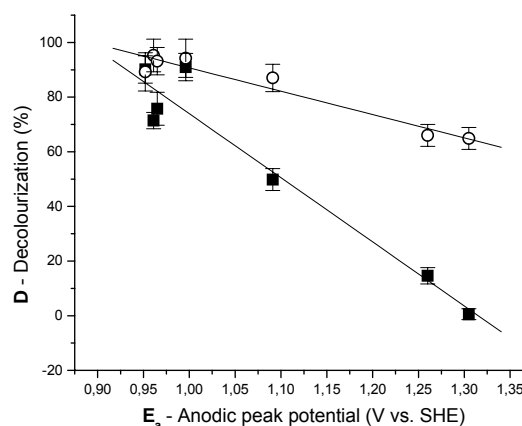
The redox peaks II<sub>a</sub> and II<sub>r</sub> can be associated with irreversible redox reactions leading to cleavage of the azo bonds. In the voltammograms of dyes VI (triazole) and VII (biazole) the number of oxidation peaks was higher than that observed for monoazo dyes. These peaks resulted from the oxidation of the amine products generated during the disruption of more than one azo bond in these dye molecules. To confirm this theory the cyclic voltammograms of the pure amine product solutions were performed separately. The results peaks could be overlaid, respectively, to the peaks I and II in the azo dye voltammograms (data not shown).

**Decolorization with Laccase.** It has been reported that the chemical structures of the dyes largely influence their decolorization rates with laccase and that its decolorization efficiency was limited to several azo dye structures (9, 23). A correlation between the enzyme redox potential and its activity

**Table 1.** Percent Decolorization with Laccase or Laccase + HBT and Oxidation Peak Potentials (vs NHE) of Tested Azo Dyes

Dye	% decolourization (± SD)		Oxidation peak <sup>a</sup> (V)
	Laccase	Laccase+HBT	
I	71 ± 3	95 ± 6	+ 0.961
II	76 ± 6	93 ± 5	+ 0.965
III	90 ± 5	89 ± 7	+ 0.952
IV	91 ± 5	94 ± 7	+ 0.996
V	15 ± 3	66 ± 4	+ 1.260
VI	50 ± 4	87 ± 5	+ 1.091
VII	0.6 ± 0.2	65 ± 4	+ 1.305

<sup>a</sup>Potentials (vs Ag/AgCl (3MNaCl) and corrected to NHE) were recorded in both laccase and laccase/mediator system without significative change in potential.



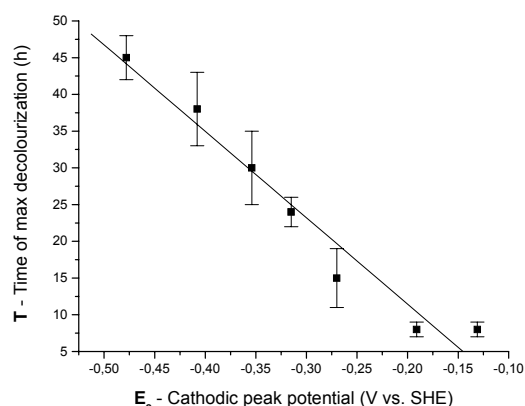
**Figure 2.** Correlation between anodic peak potential ( $E_a$ ) and decolorization % of azo dyes after 1 h with (■) laccase and (○) laccase/HBT mediator system. Correlation:  $D$  (■) =  $(308.6 \pm 28.9) - (234.6 \pm 26.6) E_a$ ,  $r^2 = 0.97$ ,  $SD = \pm 9.7$ ;  $D$  (○) =  $(176.1 \pm 10.8) - (85.4 \pm 9.9) E_a$ ,  $r^2 = 0.97$ ,  $SD = \pm 3.6$ .

toward the substrates has also been described (24, 25). The driving force for a redox reaction is expected to be proportional to the difference between the redox potentials of oxidant and reductant. For laccase-mediated oxidations, an increase in the substrate redox potential should therefore decrease the efficiency of the reaction. This hypothesis was tested by measuring the percentage of decolorization of each dye in the presence of laccase alone or laccase + HBT after 1 h of incubation. The observed results are summarized in Table 1, together with the respective anodic peak potential (26). The potential of the anodic peak gives the “degradation potential” where the degradation occurs in an irreversible redox reaction. As seen in Figure 2, a remarkably good linear correlation was found, in both systems, between the percentage decolorization of each dye and the respective anodic peak potential. The linear relationship was preserved for up to 2 h, during the initial period of decolorization. When the maximum of decolorization was reached, the linearity disappeared. An important observation is that the anodic peak potentials of all the dyes are higher than

**Table 2.** Times of Maximum Decolorization ( $\geq 98\%$ ) by Yeast Strain *I. occidentalis* and Reduction Peak Potentials (vs NHE) of Tested Azo Dyes

dye	time (h) for max decolourization $\geq 98\%$ ( $\pm$ SD)	Reduction peak <sup>a</sup> (V)
I	$8 \pm 1$	-0.191
II	$8 \pm 1$	-0.131
III	$24 \pm 3$	-0.315
IV	$30 \pm 4$	-0.354
V	$15 \pm 2$	-0.270
VI <sup>b</sup>	$38^2 \pm 5$	-0.408
VII <sup>c</sup>	$45^3 \pm 5$	-0.478

<sup>a</sup>Potentials were recorded vs Ag/AgCl (3M NaCl) and corrected to NHE. <sup>b</sup>Concentration 0.97 mM. <sup>c</sup>Concentration 1.01 mM.

**Figure 3.** Correlation between cathodic peak potential ( $E_c$ ) and time of max % decolorization of dyes. Correlation:  $T$  (■) =  $(12.1 \pm 3.7) + (-117.6 \pm 11.3) E_c$ ,  $r^2 = 0.97$ ,  $SD = \pm 3.3$ .

the reported redox potential for *T. villosa* laccase (+0.780 V vs NHE) and, even so, most of them were extensively decolorized by laccase. The exceptions were dyes V and VII, for which were found high oxidation potentials (Table 1). These facts can be understood in the light of the Nernst equation. Any redox reaction is dependent on the formal redox potential and on the concentrations of the reduced and oxidized species. The laccase dye ratio in our system is about 1:1000 or more to the dye. These concentration differences can explain why laccase can oxidize such dyes high degradation potential.

Concerning the positive effect of HBT on the decolorization degree, this can be rationalized considering that the laccase/HBT system, which is also effective through the formation of a free radical, is a stronger oxidant than laccase itself (+1.084 V vs NHE) (26). Thus in the oxidative dye decolorization approach using laccase or laccase/mediator, the redox potential difference between the biocatalyst and the dye is a relevant indicator whether the enzyme is able to decolorize the dye.

**Decolorization by *I. occidentalis*.** Ionizable azo dyes are impermeant to cell membranes and their transformation by living microbial cells must thus occur in the extracellular medium (27). Azo dyes can

be reduced by two or four electrons to produce usually colorless hydrazo compounds or amines, respectively (28). In the case of bisazo dyes the reduction of the azo bonds occurs consecutively (29). The substituents next to the azo bond affect the rate of azo dyes reduction (30). The process is also facilitated by redox mediators (31). Previous work with yeasts has shown that azo dyes are reduced to amines (19). In this work we investigated the possibility of using data obtained by cyclic voltammetry to predict relative decolorization rates of azo dyes by *I. occidentalis*. Our approach was to measure the times required for  $\geq 98\%$  decolorization of the dyes (Table 2). As seen in Figure 3, an approximately linear correlation was observed between the decolorization times and the cathodic peak potentials of the tested dyes. Concerning cell-mediated reductions, NAD(P)H is generally assumed to be the primary electron donor. The driving force for the reduction reactions promoted by NAD(P)H will therefore be proportional to the difference between the reduction potentials of the donor and acceptor species: the less negative the redox potential of the azo dye, the more favorable (and faster) will be its reduction (32, 33). We confirmed these principles in our observations.

## Conclusions

We found a linear relationship during the initial period of decolorization with laccase and a laccase/mediator system between the percentage decolorization of each dye and the respective anodic peak potential.

Contrarily to the laccase system, *I. occidentalis* decolorizes azo dyes through a reductive mechanism, but also in this system a linear relationship between the cathodic peak potentials and the time of maximum decolorization of the azo compounds was observed.

The redox potential differences between the biocatalysts and the dyes is a relevant indicator whether the enzyme is able to decolorize the dye.

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## **CHAPTER 5**

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### **FRE1P AND THE AZOREDUCTASE ACTIVITY OF *SACCHAROMYCES CEREVISIAE***

## Azo reductase activity of intact *Saccharomyces cerevisiae* cells is dependent on the Fre1p component of the plasma membrane ferric reductase

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### Abstract

Unspecific bacterial reduction of azo dyes is a widely studied process in correlation with the biological treatment of coloured waste waters but the enzyme system associated to this bacterial capability has never been positively identified. Several ascomycete yeast strains display similar decolourising behaviour. The yeast-mediated process requires an alternative carbon and energy source and is independent on previous exposure to the dyes. When substrate dyes are polar their reduction is extracellular, strongly suggesting the involvement of an externally-directed plasma membrane redox system. The present work demonstrates that, in *Saccharomyces cerevisiae*, the ferric reductase system participates in the extracellular reduction of azo dyes. The *S. cerevisiae* mutant strains  $\Delta fre1$  and  $\Delta fre1\Delta fre2$ , but not  $\Delta fre2$ , showed a much reduced decolourising capability. *FRE1* gene complemented the phenotype of *S. cerevisiae*  $\Delta fre1$  cells recovering the ability to grow in medium without externally added iron and to decolourise the dye, following a pattern similar to the one observed in the wild-type strain. These results suggest that under the conditions tested, Fre1p is a major component of the azo reductase activity.

### INTRODUCTION

Research work on biodegradative processes of azo dyes usually exploits bacterial species, either isolated or in consortia (4,36). Bacteria, under appropriate conditions (oxygen limitation, presence of substrates utilized as carbon and energy sources) frequently reduce azo dyes, producing colourless amines. Nevertheless many dyes are recalcitrant to conventional wastewater treatment processes by activated sludge (4). The overall impression on this research area is that many azo dyes can be reduced (and decolourised) by a considerable number of bacterial species but, as far as we know, the enzyme responsible for the unspecific primary reduction step has never been positively identified. What is currently postulated is that reductive decolourisation of sulfonated azo dyes by living cells must occur extracellularly due to the impermeant nature of those compounds, and that the primary reductant is a cytoplasmic electron donor, presumably NAD(P)H (36).

Our own studies (30,31) have demonstrated that some non-conventional ascomycete yeasts are efficient azo dye decolourisers acting, as many bacteria, by reducing the azo bond. Dye decolourisation by yeasts is comparatively unspecific, but is affected by the medium

composition, by the used yeast strain, and by parameters as pH and dissolved oxygen. It also depends on actively growing cells, being faster during the exponential growth phase, and displays an enzyme-like temperature profile, strongly suggesting its biotic nature. However, further information is required for a successful application of yeasts in a wastewater treatment process. The present work was developed to demonstrate the participation of an externally directed plasma membrane redox system (PMRS) in azo dye reduction, linking an intracellular reductant to an extracellular electron acceptor. As a required first step, it was necessary to find a model yeast strain capable of decolourising polar azo dyes. Among the screened strains, *Saccharomyces cerevisiae* CEN.PK113-7D proved to fulfil those conditions.

In *S. cerevisiae* the most extensively explored PMRS (plasma membrane redox system) is the ferric/cupric reductase system which participates in the high-affinity uptake of iron. This activity can be assayed through the reduction of impermeant substrates like ferricyanide, iron(III)-citrate, iron(III)-EDTA, and a variety of other ferric chelates. In this complex system the best studied components are the metallo-reductases encoded by the genes *FRE1* (7) and *FRE2* (15), the *FET3/FTR1* encoding the oxidase-permease complex (reviewed in 9), the iron-dependent transcriptional regulators Aft1p (39,41) and Aft2p (3,40) and the copper-dependent transcriptional regulator Mac1p

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(16,40). A potential  $\text{Fe}^{3+}/\text{Cu}^{2+}$  reductase subunit is the cytoplasmic cofactor Utr1p (1).

*FRE1* and *FRE2* encode plasma membrane proteins (7,15) and are both transcriptionally activated by Aft1p, whose intracellular location is dependent on iron (III) level (42). *FRE1* activation is also controlled by Aft2p (33) and Mac1p (40). Transcription of *FRE2* depends only on iron levels (14) through Aft1p (33). The protein encoded by *FRE1* contains several transmembrane domains (7), and shares a 62% sequence similarity with the gp91<sup>phox</sup> subunit of cytochrome b<sub>558</sub> (32). The protein motifs in gp91<sup>phox</sup> responsible for binding FAD and NADPH are conserved in Fre1p (12,23,35). Fre1p and Fre2p together account for virtually all of the  $\text{Fe}^{3+}/\text{Cu}^{2+}$  reductase activity of yeast cells but in varying proportions, depending both on iron and/or copper availability and on the growth phase of the cells (14,15,16). Typically *FRE2* is induced at a later stage. Fre1p and (or) Fre2p reduce external  $\text{Fe}^{3+}$  (or  $\text{Cu}^{2+}$ ) prior to their uptake, mediated by Fet3p/Ftr1p, where Fet3p is a multicopper oxidase and Ftr1p the permease component (10). The cytoplasmic cofactor Utr1p in *S. cerevisiae* has recently been shown to be a NAD kinase (21) which is regarded as the only enzyme catalysing the synthesis of NADP.

The genome sequence of *S. cerevisiae* revealed the presence of five additional metalloregulated genes, *FRE3-FRE6* and *FRE7*, with sequence similarities to *FRE1* and *FRE2*. The first four are transcriptionally regulated by the iron-responsive Aft1p element and the fifth by the copper-dependent Mac1p (27). Fre3p and Fre4p are potential siderophore-iron reductases (43), but the function of the remaining genes is unknown. Given their regulation pattern they may participate in iron homeostasis (*FRE5*, *FRE6*) and copper homeostasis (*FRE7*), possibly as internal metalloreductases (27).

The present work shows that the azoreductase and ferric reductase activities of yeast cells assayed in different growth phases are closely parallel, being at the highest level during the late exponential growth phase. This property of ferric reductase has been described in early studies (6,15). Also, deletion of the *FRE1* gene eliminates a major fraction of the azoreductase activity in intact cells of *S. cerevisiae* harvested in the late exponential growth phase, whereas the deletion of the *FRE2* gene has a minor effect on that activity. We believe that our results will be relevant for biotechnological applications of this activity and also for a broader understanding of the unspecific redox activities associated to the yeast plasma membrane.

## MATERIALS AND METHODS

**Chemicals.** The azo dye used in the experiments was *m*-[(4-dimethylamino)phenylazo] benzenesulfonic acid, sodium salt, and was synthesized as described for methyl orange (13).

**Yeast strains and plasmids.** The yeast strains and the plasmids used in this work are listed respectively in tables 1 and 2. The cultures were maintained on slants of YPD - yeast extract (1% w/v), peptone (1% w/v),

glucose (2% w/v) and agar (2% w/v). Growth on solid media was carried out at 30°C.

TABLE 1  
*Saccharomyces cerevisiae* strains used in this work

Strain	Genotype of interest	Reference
CEN.PK 113-7D	wt ( <i>MATa</i> , <i>MAL2-8c</i> <i>SUC2</i> )	11
Y04163	BY4741; <i>Mat a</i> ; <i>his3</i> ; <i>leu2</i> ; <i>met15</i> ; <i>ura3</i> ; <i>YLR214W::KanMX4</i>	Euroscarf
Y07039	BY4741; <i>Mat a</i> ; <i>his3</i> ; <i>leu2</i> ; <i>met15</i> ; <i>ura3</i> ; <i>YKL220C::KanMX4</i>	Euroscarf
SP1	$\Delta fre1$ (CEN.PK <i>YLR214W::KanMX4</i> )	This work
SP2	$\Delta fre2$ (CEN.PK <i>YKL220C::KanMX4</i> )	This work
SP3	BY4741; <i>YKL220C::HphMX4</i>	This work
SP4	$\Delta fre1\Delta fre2$ (CEN.PK <i>YLR214W::KanMX4</i> <i>YKL220C::HphMX4</i> )	This work
SPcmp- <i>FRE1</i>	$\Delta fre1$ (pSP3) (CEN.PK <i>YLR214W::KanMX4</i> + plasmid pSP3)	This work

TABLE 2  
*Plasmids used in this work*

Plasmids	Reference
pSP1 ( <i>FRE1</i> in pGEM)	This study
pSP2 ( <i>FRE2</i> in pGEM)	This study
pAG32	19
pSH65	20
pSP3 ( <i>FRE1</i> in pSH65)	This study

**Cell growth in liquid medium.** The attenuation of appropriately diluted cell suspensions (as described in 30) was measured at 640 nm in a Spectronic 21 Bausch & Lomb using a 1 cm path length cell.

**Decolourisation in liquid media.** Decolourisation experiments by growing cultures of *S. cerevisiae* CEN.PK113-7D (also reported as wt strain along this work) were typically performed in 250 mL cotton-plugged Erlenmeyer flasks with 100 mL of sterile medium (normal decolourisation medium, here referred to as NDM) containing yeast extract (0.25%, w/v), glucose (2%, w/v) and 0.2 mmol.L<sup>-1</sup> of the tested dye in a mineral salts base of the composition previously described (31) incubated at 26°C and 120 rpm. Whenever required, iron (III) was added to medium as the EDTA chelate, from a 100 mM stock solution in FeCl<sub>3</sub> and EDTA. For the mutant strains, which show impaired growth in our standard medium, cells were grown for 137 h on NDM supplemented with 2 mM iron (III) as the EDTA chelate. For control wild-type cells were grown in similar conditions. The cells were then harvested by centrifugation at 16.1xg, washed several times with sterile distilled water, and



resuspended on NDM to produce cell suspensions with  $3.8 \pm 0.2$  attenuation units ( $4.2 \pm 0.2$  g.L<sup>-1</sup> cell dry weight). Throughout this work, decolourising activity refers to the decolourisation capability of growing yeast cultures.

**Cell counting.** Cell suspensions (diluted to an attenuation of c.a. 0.5 units) were diluted 1:25000 and 1:250000. From each dilution 100  $\mu$ L was spread in YPD agar plates. The plates were incubated at 37°C for 2 days and after that time the number of isolated colonies was counted. All plates with more than 300 colonies or less than 30 were not considered. All the dilutions were prepared in triplicate.

**Ferric reductase assay.** Cells were grown for c.a. 6 hours in NDM, harvested by centrifugation, washed twice with sterile distilled water and resuspended in assay buffer, consisting of 0.05 M sodium citrate pH 6.5 with 5% glucose, at a density of c.a.  $1.3 \pm 0.1$  attenuation units ( $1.4 \pm 0.1$  g.L<sup>-1</sup> cell dry weight). The assays were performed in triplicate at two different cell densities obtained with either 780  $\mu$ L of suspension or 390  $\mu$ L of suspension plus 390  $\mu$ L of assay buffer. The cell suspensions were pre-incubated for 10 min at room temperature. The final assay mixtures contained, in a total volume of 1 mL, 2 mM ferrozine ([3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine]) and 0.2 mM iron(III) as ferric chloride. The mixtures were allowed to react at room temperature ( $20 \pm 2^\circ\text{C}$ ) for 5 or 10 min. Cells were then harvested by centrifugation and the optical density at 562 nm was measured against a blank prepared similarly but without cells. The ferrous iron concentration was estimated by using a molar absorbance of 27900 M<sup>-1</sup>cm<sup>-1</sup> for the iron(II)-ferrozine complex (17).

**Azoreductase assays.** These assays were performed as the ferric reductase assays but using acetate buffer 0.05 M pH 4.0 and 5% glucose. The assay mixture contained a cell suspension of 1 or 2 attenuation units ( $1.1 \pm 0.1$  or  $2.2 \pm 0.1$  g.L<sup>-1</sup> cell dry weight) and 0.05 mM dye, and was allowed to react for 15 to 20 min. Within this period the decrease in absorbance was linear with time. The optical density of the final supernatants was read at dye  $\lambda_{\text{max}}$  (461 nm). The amount of dye reduced was determined from a molar absorbance of 21440 M<sup>-1</sup>cm<sup>-1</sup>, obtained from a calibration curve. Throughout this work, azo reductase specific activity refers to the results of activity assays within a short period of time, being expressed as  $\mu\text{mol} \cdot (\text{g cell dry weight} \cdot \text{min})^{-1}$ .

**Transformation of *S. cerevisiae* cells.** Transformation of *S. cerevisiae* cells was done by the LiAc/SS-DNA/PEG method (18). When required transformants were recovered at 30°C in YPD medium for 4 hours before plating on YPD solid medium containing either 200 mg.L<sup>-1</sup> geneticin (G418 from Life Technologies) or 30  $\mu\text{g} \cdot \text{mL}^{-1}$  phleomycin (CAYLA, Toulouse, France). Transformants were obtained after 2-3 days of incubation at 30°C. To purify transformants from background each large colony was re-streaked on fresh YPD-geneticin or YPD-phleomycin plates. Only those clones that grew after the double selection were further analysed as potentially correct transformants, by analytical PCR as described by Kruckeberg (22).

**Cloning of the *FRE1* and *FRE2* genes.** *FRE1* gene was amplified by PCR with the Pfu Turbo DNA polymerase (Stratagene), using the primers Fre1forw and Fre1rev and genomic DNA isolated from *S. cerevisiae* CEN.PK. The PCR fragment was cloned into the plasmid pGEM<sup>®</sup>-T Easy vector (PROMEGA), originating the plasmid pSP1 (table 2). The primers Fre2forw and Fre2rev were used to amplify *FRE2* gene, following the same procedure as described for *FRE1* gene. The PCR product was cloned in pGEM<sup>®</sup>-T Easy vector originating the plasmid pSP2 (table 2). DNA cloning and manipulation were performed according to standard protocols (34).

***FRE1* knock-out.** The *S. cerevisiae* Y04163 strain deleted in the gene *FRE1* (*YLR214W*) was obtained from the Euroscarf collection. Two primers, A-YLR214W and D-YLR214W (table 3) were used to amplify by PCR the *YLR214W::KanMX4* allele of the *S. cerevisiae* strain Y04163. The PCR product was used to transform wt cells. Cells were plated on YPD solid medium containing 200 mg.L<sup>-1</sup> geneticin. Successful integration of the *YLR214W::KanMX4* cassette was scored by presence of the *YLR214W::KanMX4* band (2352bp) and absence of the *YLR214W* wild-type band (2796bp) following analytical PCR on whole cells using the same primers. Internal primers to the kanamycin cassette (K2 and K3, see table 3) were also used to reconfirm the disruption. This strain was named SP1.

***FRE2* knock-out.** The procedure followed to disrupt the gene *FRE2* (*YKL220C*) was similar to the one described above. Primers, A-YKL220C and D-YKL220C (table 3) were used to amplify by PCR the *YKL220C::KanMX4* allele in the *S. cerevisiae* strain Y07039. The PCR product was used to transform the *S. cerevisiae* CEN.PK strain and correct integration of the cassette was scored by presence of the *YKL220C::KanMX4* band (2323bp) and absence of the *YKL220C* wild-type band (2842bp) following analytical PCR on whole cells using the same primers. This strain was named SP2.

***FRE1/FRE2* double knock-out.** The vector pAG32, containing the hygromycin resistance gene *HphMX4*, was digested with the restriction enzymes *Bgl*III and *Eco*RV. The digested DNA was used to switch the selective marker of the gene replacement cassette in *S. cerevisiae* Y07039 from *KanMX4* to *HphMX4*, resulting in strain SP3. The replacement of the *KanMX* for the *HphMX4* was confirmed with PCR. SP3 chromosomal DNA was used to amplify the *YKL220C::HphMX4* cassette, which was used to transform the SP1 (already carrying the *YLR214W::KanMX4*) resulting in the double mutant, SP4.

**RNA analysis.** Total cellular mRNA was prepared from yeast cells grown for 6 hours in NDM, electrophoresed on 1.5% (w/v) agarose MOPS-formaldehyde gels (29) and blotted to nylon membranes by vacuum transfer. Hybridisation was carried out using a fragment of 718 bp *Pst* I from pSP1 as a probe for *FRE1* or a fragment of 682 bp *Hind*III from pSP2 as a probe for *FRE2*. The probes were labelled according to standard procedures (34). Densitometer scanning was performed using the

Integrated Density Analysis program from the EagleSight® Software, version 3.2 (Stratagene, CA).

TABLE 3  
*Oligonucleotides used for cloning, gene deletion and verification by PCR*

Name	Sequence
A-YLR214W	AAAAATGTATTTAGGTTGCTTGACG
D-YLR214W	TATGAATTAAGGTTAGTGACGAGGC
A-YKL220C	ACAGGAAAACAAGTAAATTTTGACG
D-YKL220C	CAATTAACGTTTCATAAAATTTGCC
Fre1forw	ATGGTTAGAACCCGTGTATTATTC
Fre1rev	TTACCATGTAAAACTTCTTC
Fre2forw	ATGCATTGGACGTCCATCTTG
Fre2rev	TCACCAGCATTGATACTCTTC
K2	CGATAGATTGTCGCACCTG
K3	CCATCCTATGGAAGTGCCTC
CMPfre1forw	CATGGATCCAAAATGGTTAGAACCC
	GTG
CMPfre1rev	CATGTCGACTTACCATGTAAAACTTT
	CTTC
GAL1p_c	ATTGTTAATATACCTCTATACTTTAAC

**Construction of the pSH65-FRE1 vector.** The ORF of *FRE1* was amplified by PCR with the primers CMPfre1forw and CMPfre1rev. CMPfre1forw contains one *Bam*HI site and the CMPfre1rev contains one *Sal*I site which were used for cloning the *FRE1* ORF in the vector pSH65 (20) using the same restriction sites. The *FRE1* ORF was directionally cloned between the GAL1,10 promoter and the CYC1 terminator in the vector pSH65, which is a CEN6/ARSH4 low-copy number vector carrying the *ble<sup>r</sup>* phleomycin resistance gene for selection in yeast. Correct clones were verified by sequencing. A clone named pSP3 (table 2) was selected for further studies.

**Transformation of *Δfre1* with the plasmid pSP3 (pSH65-FRE1).** Cells of the strain SP1 were transformed with the plasmid pSP3 and plated on YPD solid medium containing 30  $\mu\text{g}\cdot\text{mL}^{-1}$  phleomycin. Ten colonies were checked by analytical PCR using the primers GAL1p\_c and CMPfre1rev. The method described by the “The SixPack Guidelines” of the EUROFAN project was used. The GAL1p\_c and the CMPfre1rev forms a 2.1 kb PCR product only if the *FRE1* ORF is present in the correct orientation with respect to the GAL1,10 promoter in pSH65. One of the positive strains was named SPcmp-FRE1 (table 1) and was used in further studies.

## RESULTS

**Decolourization by growing yeast cultures.** Growing cultures of *S. cerevisiae* completely decolourised the tested azo dye in c.a. 8.5 h. Figure 1A illustrates the yeast cells growth curve, and the pH variation and dye absorbance in the supernatant medium. A diauxic growth was observed, with a specific growth rate of 0.175  $\text{h}^{-1}$ , when growing in glucose, and of 0.013  $\text{h}^{-1}$  after switching to ethanol utilization. The decolourisation progress was unaffected by previous exposure of the cells to the dye (results not shown). Similar observations have been described earlier for *Candida zeylanoides* (31) and *Issatchenkia occidentalis* (30). The confirmation that colour loss was due to the reductive cleavage of the azo bond in the dye molecules was provided by the detection of the related aromatic amines by HPLC analysis, as shown in a previous work (31).

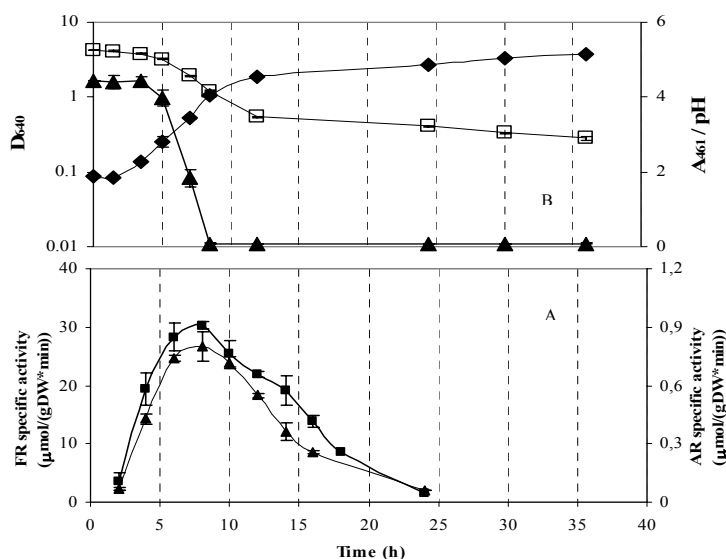


Figure 1. **Decolourisation progress and effect of growth stage on ferric reductase and azo reductase specific activities.** (A) Time course of cell growth, measured as attenuation at 640nm ( $D_{640}$ ; ♦), pH variation (pH; □) and progress of decolourisation, measured as dye absorbance at 461 nm ( $A_{461}$ ; ▲). *S. cerevisiae* was grown at 26°C and 120 rpm, in normal decolourisation medium containing 0.2mM dye. (B) Variation of ferric reductase (FR; ■) and azo reductase (AR; ▲) specific activities in cells of *S. cerevisiae* harvested at the specified times, expressed as  $\mu\text{mol} \cdot (\text{g cell dry weight})^{-1} \cdot \text{min}^{-1}$ . The cells were grown in normal decolourisation medium at 26°C and 120 rpm.

The effect of the growth phase on specific ferric and azo reductase activities was determined by assaying cells harvested from growing cultures at different incubation times. The results are shown in figure 1B, and despite the difference in the absolute values, the two curves are closely parallel at all times. Both have an activity peak in the late exponential growth phase, which is also when the fastest decrease of dye concentration in the incubation medium is observed.

**Effect of iron concentration on specific ferric and azo reductase activities.** The progress of decolourisation by growing cultures was measured in incubation media with different iron (III) concentrations, supplied as the EDTA chelate. Increasing iron concentrations resulted in a much delayed decolourisation. As seen in figure 2A, total decolourisation required over 50 h in the presence of 1.0 mM iron (III), in contrast with the 8.5 h required in NDM without iron addition. In media containing 2.5mM iron(III) dye concentration decreased only *c.a.* 20% in 50h. For concentrations above 2.5mM iron(III) we observed precipitation of the iron in the medium. The reduced decolourising activity of the cells grown at higher iron concentrations was not due to impaired growth or loss of cell viability since cell counting in aliquots of the different cultures, collected after 28 h of growth, produced identical numbers of viable cells. Azo and ferric reductase activities were also measured in cells harvested from growth media with different iron concentrations, after 6 hours of growth. Cells were collected at this point because of the peak activity of both enzymes around this time. The results in Figure 2B show that the production of both activities was repressed by iron, in a concentration-dependent manner: azo reductase activities are reduced to *c.a.* 20% at 1 mM iron and to 2% at 2.5 mM iron, despite the growth stimulation at higher Fe

concentrations (data not shown). These observations point to an additional link between these two activities.

**Effect of deletions of *FRE1* and *FRE2* genes on the activities of ferric and azo reductases.** The mutant strains of *S. cerevisiae*  $\Delta fre1$ ,  $\Delta fre2$  and  $\Delta fre1\Delta fre2$  have impaired growth in iron-deficient media. In order to overcome this problem, decolourisation assays with the mutant strains were performed at high density suspensions of pre-grown cells, as described in Materials and Methods. Under these conditions both the wt strain and the  $\Delta fre2$  mutant achieved complete decolourisation in *c.a.* 5 hours. Therefore deletion of the *FRE2* gene has a negligible effect in the decolourisation process in our experimental conditions. In contrast, the  $\Delta fre1$  and  $\Delta fre1\Delta fre2$  strains showed a much reduced decolourising activity, requiring more than 45 hours to completely remove the colour from the medium (figure 3A). The azo reductase activity assays with the different strains allowed similar conclusions. As seen in figure 3B, the specific activity in the  $\Delta fre2$  mutant reached the same order of magnitude (as compared to the wild type), whereas those in  $\Delta fre1$  and  $\Delta fre1\Delta fre2$  strains were negligible. The ferric reductase assays produced very similar results, as seen in figure 3B. These results demonstrate the importance of the *FRE1* gene product in the decolourising activity of the yeast cells.

***FRE1* expression in *S. cerevisiae*.** The expression of *FRE1* was followed by Northern-blot analysis (figure 4). In cells of wild-type strain *S. cerevisiae* CEN.PK, grown in the absence of added iron, a strong mRNA signal against a *FRE1* probe was revealed, proving the expression of this gene. Wild-type cells, grown in the presence of added iron showed decreased *FRE1* mRNA levels with increasing iron concentration in the range

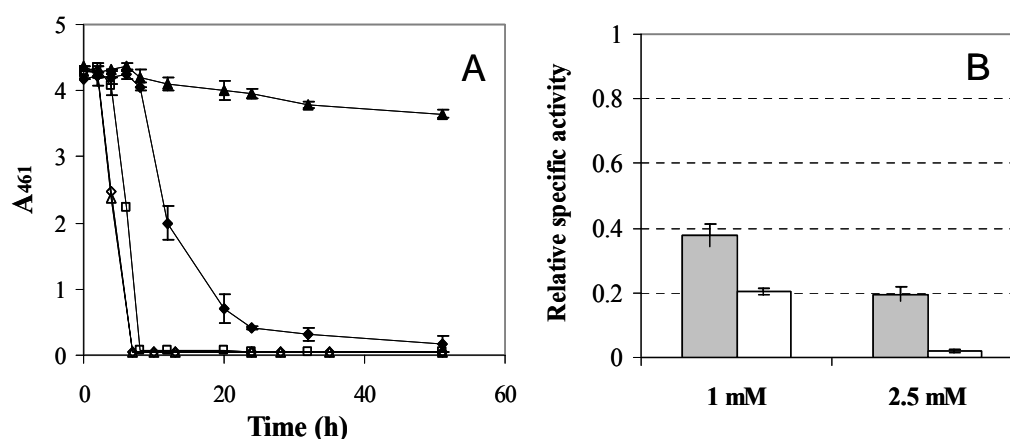


Figure 2. **Iron(III)-dependent decolourisation and activities of ferric reductase and azo reductase.** (A) Time course of dye decolourisation in the presence of 1.0mM (♦) and 2.5mM (▲) iron (III). Cells were grown at 26°C and 120 rpm in normal decolourisation medium with 0.2mM dye and iron was supplied as the EDTA chelate to the specified concentrations. Control experiments were performed without iron addition to the medium (□) and in media supplemented with EDTA, either at 1mM (◇) or 2.5mM (△). The effect was followed by measuring dye absorbance at 461 nm (A<sub>461</sub>). (B) Specific activity assays of ferric reductase (grey bars) and azo reductase (white bars) were performed with cells harvested after 6 hours growth on normal decolourisation medium at 26°C and 120 rpm. Growth media contained either 1.0mM or 2.5mM iron(III). Specific activities were calculated relative to cells grown without additional iron(III). Activities were calculated relative to cells grown without additional iron(III). Error bars are the standard deviation from three independent determinations.

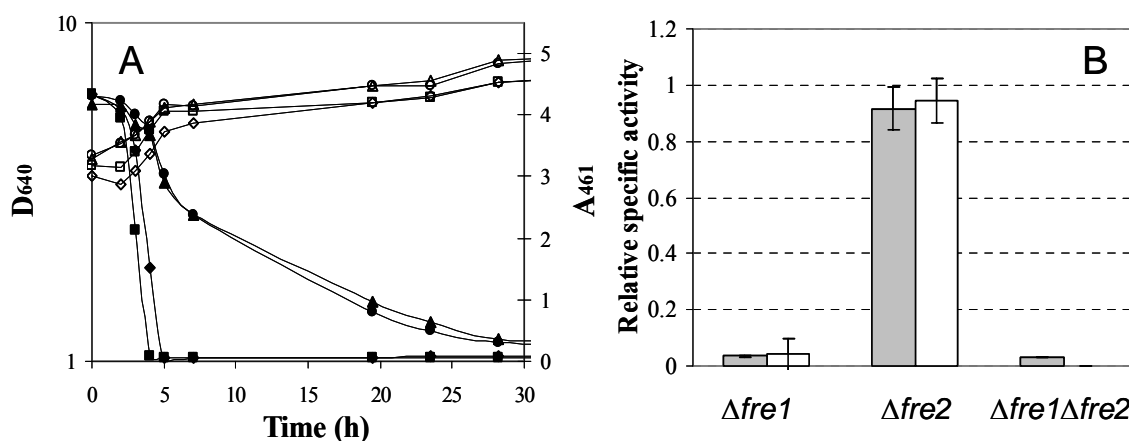


Figure 3. **Deletion of *FRE1* and *FRE2* genes affects decolourisation progress and ferric reductase and azo reductase activities.** (A) Cells were grown at 26°C and 120 rpm on normal decolourisation medium with 0.2mM dye. Cell growth was measured as attenuance at 640nm ( $D_{640}$ ; open symbols) and decolourisation progress was assessed by dye absorbance at 461 nm ( $A_{461}$ ; closed symbols): wild type ( $\diamond$ ,  $\blacklozenge$ ),  $\Delta fre1$  ( $\triangle$ ,  $\blacktriangle$ ),  $\Delta fre2$  ( $\square$ ,  $\blacksquare$ ) and  $\Delta fre1\Delta fre2$  ( $\circ$ ,  $\bullet$ ). (B) Activities of the ferric reductase (FR; grey bars) and azo reductase (AR; white bars) of *FRE* mutant strains were calculated relative to cells of the reference strain, all grown on NDM at 26°C and 120 rpm and harvested after 6 hours growth.

between 1.0 and 2.5 mM. Therefore, iron seems to regulate the expression of *FRE1* gene. As expected, in cells of *S. cerevisiae*  $\Delta fre1$  and  $\Delta fre1\Delta fre2$  deletion strains, no *FRE1* mRNA was detected.

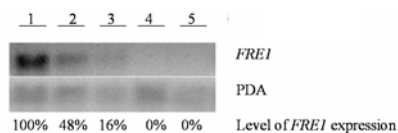


Figure 4. **Northern blot analysis of *FRE1* transcriptional level.** Cells used for RNA extraction were harvested after 6h growth on normal decolourisation medium at 26°C and 120 rpm, with or without iron addition. Each lane contained 20  $\mu$ g of total RNA and *PDA1* (38) served as internal standard. Lane 1-wt; lane 2- wt with 1mM iron(III) added to the growth medium; lane 3- wt with 2.5mM iron(III) added to the growth medium; lane 4-  $\Delta fre1$ ; lane 5-  $\Delta fre1\Delta fre2$ . The percentage of *FRE1* expression (average of two independent experiments) is relative to wt strain, grown in normal decolourisation medium without externally added iron.

**Recovery of the *FRE1* activity.** To confirm that in our experimental conditions the recovery of the azoreductase activity is mainly associated with *FRE1*, the progress of decolourisation was followed in cultures of the strains wt,  $\Delta fre1$  and  $\Delta fre1$  transformed with the plasmid pSP3 containing *FRE1* under the promoter GAL1,10. The cells were grown in media with 20g.L<sup>-1</sup> galactose as carbon source, for activation of the GAL1,10 promoter. As seen in figure 5, *FRE1* gene complemented the phenotype of *S. cerevisiae*  $\Delta fre1$  cells recovering the ability to grow in medium without externally added iron, following a pattern similar to the one observed in the wt strain. In this assay the wt and  $\Delta fre1$  strains behaved as expected regarding the ability of decolourisation, with a total removal in the wt and a negligible removal in the mutant strain. The transformed strain  $\Delta fre1$ (pSP3), although with a small delay in the starting of the decolourisation, was able to

fully decolourise the dye. This small difference could be due to distinct regulatory properties of the two promoters. These experiments provide the evidence that *FRE1* is responsible for the azo reductase activity of the intact yeast cells in our operational conditions.

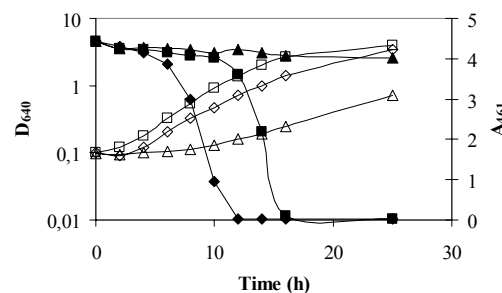


Figure 5. **Reconstitution of the *FRE1* activity.** Cells were grown at 26°C and 120 rpm on normal decolourisation medium with 0.2mM dye and with 20g.L<sup>-1</sup> galactose as carbon source for activation of the GAL1,10 promoter. Cell growth was measured as attenuance at 640nm ( $D_{640}$ ; open symbols) and decolourisation progress was assessed by dye absorbance at 461 nm ( $A_{461}$ ; closed symbols): wild type ( $\diamond$ ,  $\blacklozenge$ ),  $\Delta fre1$  ( $\triangle$ ,  $\blacktriangle$ ) and SPcnp-*FRE1* ( $\square$ ,  $\blacksquare$ ).

## DISCUSSION

Plasma membrane redox systems are ubiquitous, being expressed in all living cells including bacteria and cyanobacteria, yeasts, algae and also in plant and animal cells (8,26). These systems are linked to several vital cellular functions, including growth control, iron uptake, apoptosis, bioenergetics, transformation and hormone responses (2,5,28). Some of these roles may be linked to the maintenance of appropriate NAD(P)<sup>+</sup>/NAD(P)H cytoplasmic ratios. In fact, an increase in the glycolytic flux, leading to an accumulation of NADH in the cytoplasm, induces an increase of PMRS activity (28). A number of such

systems has been described, such as NADH:ascorbate free radical oxidoreductase, NADH:ubiquinone oxidoreductase and ferric reductase, among others (26,28). However it is not clear whether different phenomenological enzyme activities correspond to different PMRS. On the contrary, it is generally accepted that several PMRS are multifunctional (5,8,28).

The *FRE1*-dependent ferric reductase activity of intact yeast cells is inversely regulated by iron(III) concentration, through the transcriptional activators Aft1p and Aft2p (33,42). Our decolourisation experiments in media containing additional iron revealed a considerable increase in the time required for complete dye removal, and a negative effect of iron(III) in the azoreductase activity of yeast cells. Ferric reductase activities also decrease, as expected, but the effect of increased iron concentration is more pronounced in the azoreductase activities.

Both ferric reductase (23) and yeast azoreductase display an activity peak in the exponential growth phase. This is not an unexpected observation since many enzymes involved in cell growth have peak activities in this phase, when concentrations of intracellular reductants are also high.

The use of the strains defective in the genes encoding for structural components of the transmembrane ferric reductase, *FRE1* and *FRE2*, unequivocally demonstrated that Fre1p is a major component of the azoreductase system. In contrast Fre2p had a reduced importance in azo reduction, at least under our assay conditions. Our observation is in agreement with works reporting that the *FRE1* gene accounts 80 to 98% of the ferric reductase activity (6,7). Nevertheless, growing cultures of the  $\Delta fre1$  strain and of the double deleted mutant still showed a low decolourising capability. A residual ferric reductase activity has been explained by postulating the existence of an excreted reductase activity (15) which, however, has never been described. An alternative explanation has been provided by Lesuisse and colleagues (25), who have shown that the excretion of anthranilic and 3-hydroxyanthranilic acids was correlated with the extracellular ferric reductase activity. Whether those or other extracellular reductants participate in azo dye reduction requires further investigation. The insignificant participation of Fre2p in the ferric and azo reductase activities measured in this work (cells harvested after 6 hours growth) is probably due to the fact that the *FRE2* gene is expressed primarily after 8-10 hours of growth, whereas the expression of *FRE1* is highest in cells grown for up to 6 hours (14). Therefore the effect of *FRE2* was not investigated at the present stage of our work.

It must be taken into account that the ferric reductase activity of intact yeast cells does not depend exclusively on one or more transmembrane proteins encoded by *FRE* genes. The *in vivo* association of the Fre1p component to the NAD phosphorylating kinase Utr1p (21) is now generally accepted, since increased ferric reductase activity is observed only when both *FRE1* and *UTR1* are overexpressed together (23). It has therefore been suggested that Utr1p is the supplier of NADP to

the ferric reductase system (26). This is also consistent with the existence of the NADPH binding motif in Fre1p (12,23,35), suggesting that NADPH is the electron donor for iron reduction.

In conclusion, this work strongly indicates that the Fre1p-dependent reductase system of the yeast plasma membrane is an important component of the azoreductase activity in intact cells of *S. cerevisiae* harvested between mid and late exponential growth phase. Further information on the azoreductase system will be provided by examining the effect of known inhibitors of the ferric reductase, by establishing the nature of the electron donor and by searching other components affecting the *in vivo* fully functional system. For example, it has been demonstrated that the ferric reductase activity in isolated plasma membranes is due to a NADPH dehydrogenase (diaphorase) activity and that Fre1p, *per se*, has no reductase activity (23). Additionally it has been shown that activation of the *in vivo* ferric reductase system requires the integrity of the ras/cAMP pathway (24). Interestingly, among several laboratory strains of *S. cerevisiae* the only strain with decolourising activity was the CEN.PK-113.7D, which has a mutation on the *CYR1* gene encoding the enzyme adenylate cyclase (37).

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## **CHAPTER 6**

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### **COMPARISON OF THE AZOREDUCTASE ACTIVITY OF THREE ASCOMYCETE YEAST STRAINS**



# Comparison of the azoreductase activities and assimilation of dye reduction products by three ascomycete yeast strains

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## Abstract

The decolourising capabilities of *Candida zeylanoides* UM2, *Issatchenkia occidentalis* PYCC5770 and *Saccharomyces cerevisiae* CEN.PK 113-7D towards several azo dyes were compared. The presence of dyes and degradation products in the growth medium did not affect nor growth nor viability of cells. The specific degradation rates obtained showed that *S. cerevisiae* is much more efficient in the decolourisation of dyes I, II and amaranth. When comparing the  $\ln$  (apparent 1<sup>st</sup> order decolourisation rate constant/specific growth rate) vs. the reduction peak potential (measured against the Ag/AgCl reference electrode), it was found that for all three strains that this relation was represented by a second order polynomial equation. Assimilation experiments of the reduction products showed that all strains are able to use the formed amines as carbon and nitrogen sources. This represents the possibility of complete mineralization of the tested azo dyes. To our knowledge this is the only report describing this feature in a single organism under the same operational conditions. The use of alternative carbon sources was also explored. All the three strains presented the same behaviour. Along with glucose, ethanol was the only substrate that allowed both growth and decolourisation.

## INTRODUCTION

Xenobiotic are compounds that do not exist as natural products and therefore contain structural elements that cannot be synthesized biochemically. During evolution of catabolic enzymes and pathways microorganisms were not exposed to these structures and have not developed the capability to use those compounds as sole sources of carbon and energy. For example sulfo and azo groups are practically unknown amongst natural products and can be considered as real xenophores (35). The azo group,  $-N=N-$ , is the most common chromophore in textile dyes and it is present in 60-70% of the textile dyestuffs produced (25). A large fraction of the dyes belonging to this class is soluble and therefore residual dyebaths are still heavily coloured.

Because dyes are designed to be resistant to oxidative transformations, which would result in colour fading, they are usually recalcitrant to conventional wastewater treatment processes (3,27,40). The electron-withdrawing properties of the azo group explain the resistance of aromatic azo dyes to the action of oxidative enzymes (1,25) our removal in textile effluents can be achieved by physical or chemical methods as adsorption, precipitation, coagulation, filtration or oxidation (for reviews on the subject see 1,29,36,41). However the operational costs of these processes are high and, additionally, they may generate high amounts of sludge and secondary pollution, due to the used chemicals (42). A promising and environment friendly alternative is the biological decolourisation of these type of wastewaters.

The decolourisation of dyes by microorganisms is commonly done with bacteria or white-rot fungi. In bacteria the mechanisms of decolourisation can be: non-specific reduction by electron transporters from the cellular metabolic pathways (flavins, quinones), dye-specific reduction by azo reductase enzymes and chemical reduction by sulphide generated in sulphate reduction (30,44). In white-rot fungi the degradation processes is connected to the lignin modifying exoenzymes: laccase, manganese peroxidase and lignin peroxidase (8,18,21,24,38). Although these microorganisms are able to decolourise dyes in liquid fermentations, enzyme production has also been shown to be unreliable (36).

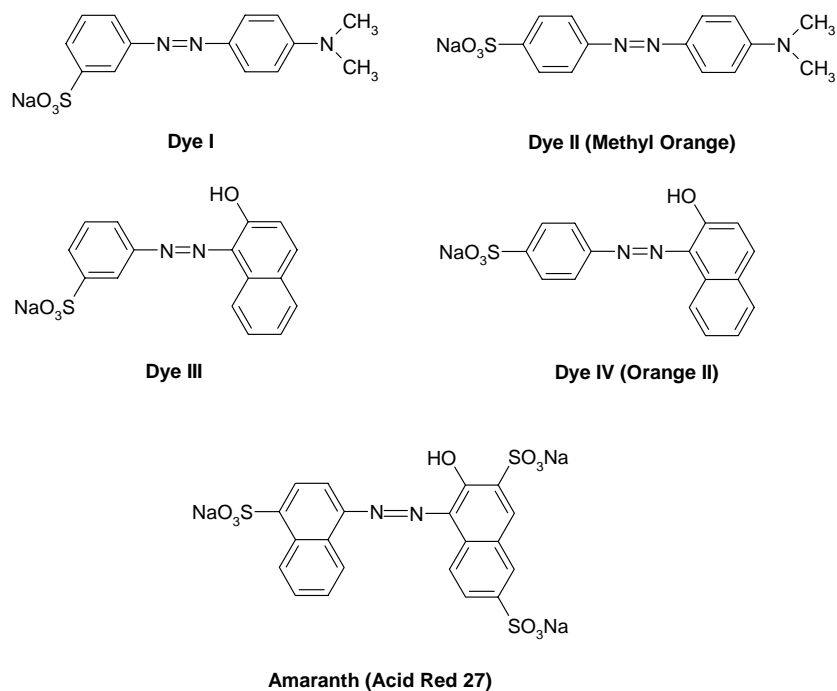
Independently on the microorganism used there are several parameters affecting the colour removal efficiency: medium composition, oxygen, temperature, pH, dye concentration, dye structure, electron donor, redox potential, redox mediators and the presence of inhibitory substances (for reviews on the subject see 18,29). For instance oxygen, due to its high redox potential, can compete with the dye for the electrons during the reduction stage and, during cell growth, will affect the physiological characteristics of the cells (17,29). Nevertheless there are reports of microorganisms capable of decolourising dyes in aerobic conditions (7,12,28,45). Temperature and pH will affect mainly the physiological state of the cells. Dyes with simple structures and low molecular weights exhibit higher rates of colour removal, whereas colour removal is more difficult with highly substituted, high molecular weight dyes (37). Inhibitory substances can be the resulting

amines or additives used in textile industry like salts, detergents and other substances (11,14). The amines that result from the reductive cleavage of the azo bond are carcinogenic and/or mutagenic and can be toxic to the decolourising microorganism (1,3,9,10,47). Salts are usually present in this type of wastewater because they are used to ensure maximum fixation of the dye to the fiber (11,22). When present in high concentrations (>1% salt) they cause plasmolysis and/or loss of cell activity (23).

In literature the ability to degrade azo dyes by yeasts was only described in a few reports (26,31,32,33) although several others exist that describe biosorption of the dyes by these organisms (2,6,15). With this work we intend to characterise and compare the azoreductase activity in three yeast strains: two isolated from contaminated soil, *Issatchenkia occidentalis* PYCC5770 (31) and *Candida zeylanoides* UM2 (26,33), and one laboratorial strain, *Saccharomyces cerevisiae* CEN.PK 113-7D (32).

## MATERIALS AND METHODS

**Chemicals and culture media components.** Dye II (Methyl Orange, CI 13025), Dye IV (Orange II, CI 15510), both *c.a.* 85% dye content, and Amaranth (Acid Red 27) were purchased from Sigma-Aldrich and used without further purification. Dyes I (*m*-[(4-dimethylamino)phenylazo]benzenesulfonic acid) and III (*m*-[(2-hydroxy-1-naphthyl)azo]benzenesulfonic acid), with a minimum 90% dye content, were synthesized by the conventional method as described previously (31). Inorganic media components, D-glucose, ethanol, sodium acetate, pyruvate, lactic acid and glycerol, of analytical grade, were obtained from Merck. Sulfanilic acid, metanilic acid, 1-amino-2-naphthol and *N,N*-dimethyl-*p*-phenylene diamine were from Aldrich. Complex media components (yeast extract, yeast carbon base, yeast nitrogen base and peptone) were obtained from Difco. The chemical structures of the dyes used in this work are depicted in Figure 1.



**Figure 1.** Structures of the azo dyes used in this work.

**Microorganism and maintenance conditions.** The ascomycete yeasts *Candida zeylanoides* UM2 and *Issatchenkia occidentalis* PYCC5770 (Portuguese Yeast Culture Collection), were isolated as described in a previous publication (33). The strain *Saccharomyces cerevisiae* CEN.PK 113-7D (32) was kindly provided by Dr. P. Kotter. The strains were routinely maintained on slants of a medium containing (% w/v): glucose (2), peptone (1), yeast extract (0.5), and agar (2).

**Analytical methods.** Biomass was measured by turbidimetry readings of appropriately diluted culture samples at 640nm, against a blank prepared with the same dilution of the supernatant in distilled water. The correlation

between  $OD$  (640nm) and cell dry weight (DW, g.L<sup>-1</sup>) is depicted in table 1, as experimentally determined by the standard gravimetric method. Dye concentration was estimated by absorbance measurements of supernatant samples, diluted as required, in a buffer of pH 4.0, at dye  $\lambda_{max}$ , read against a blank containing the same dilution of buffer in water (33).

**Table 1.** Relation between optical density read at 640 nm ( $OD_{640\text{ nm}}$ ) and cell dry weight (DW) of the yeast strains used.

Strain	Relation between OD and cell dry weight (DW)
<i>I.occidentalis</i> PYCC5770	$DW(g.L^{-1}) = 1,1592 * OD_{640\text{ nm}} - 0,0106, r^2=0,9971$
<i>C.zeylanoides</i> UM2	$DW(g.L^{-1}) = 1,1067 * OD_{640\text{ nm}} - 0,0207, r^2=0,9955$
<i>S.cerevisiae</i> CEN.PK 113-7D	$DW(g.L^{-1}) = 1,1013 * OD_{640\text{ nm}} - 0,013, r^2=0,9959$

**Growth and decolourisation assays.** Decolourisation experiments by growing cultures were typically performed in 250 mL cotton-plugged Erlenmeyer flasks with 100 mL of sterile medium (normal decolourisation medium, here referred to as NDM) containing yeast extract (0.25%, w/v), glucose (2%, w/v) and 0.2 mmol.L<sup>-1</sup> of the tested dye in a mineral salts base of the composition previously described (33) incubated at 26°C and 120 rpm. To test alternative carbon sources NDM was prepared without glucose and at a double concentration. Solutions (%w/v) of sodium acetate (13), starch (1), glucose (20), piruvate (5), glycerol pH 5.5 (10) and lactate pH 5.5 (1) were prepared, filter sterilised and aseptically added to the medium to the desired final concentrations (0.5%). Concentrated azo dye solutions (4mM) were separately prepared and filter sterilized (Filtropur 0.2µm) and aseptically added to the medium to a final concentration of 0.2 mM. The flasks were inoculated with a cell suspension obtained from a freshly grown slant, and incubated at 26°C, under orbital shaking (120 rpm). Triplicate experiments were run throughout.

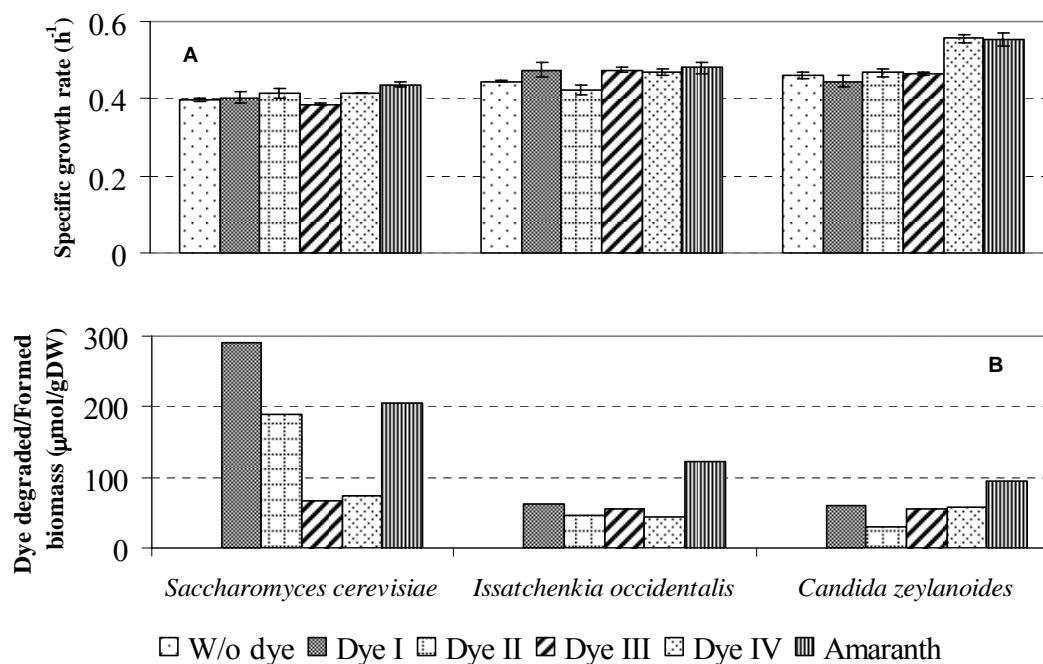
**Assimilation experiments.** Minimal media (yeast nitrogen base, YNB, and yeast carbon base, YCB) were prepared according to the manufactor's instructions and supplemented with 0.5mM metanilic acid, sulfanilic acid, 1-amino-2-naphtol or N,N-dimethyl-p-phenylene diamine for testing these compounds as carbon and energy or nitrogen sources. Control experiments were performed with 0.5mM ammonium sulphate as sole nitrogen source, in YCB, or with 0.5mM glucose as sole carbon and energy source, in YNB. These experiments were performed in liquid and in solid (2% agar w/v) media.

**Cyclic voltammetry experiments.** All voltammetric measurements were performed using a Voltalab 30 potentiostat (Radiometer Analytical, France) controlled by a personal computer running Voltamaster 4 electrochemical software. The working electrode was a glassy carbon electrode with a geometric area of 0.07 cm<sup>2</sup> (BAS, USA). The counter electrode electrode was a coiled platinum wire electrode with a length of 23 cm (BAS) and the reference electrode a silver/silver chloride electrode filled with 3 M sodium chloride (Ag|AgCl (3M NaCl)) (BAS). The glassy carbon electrode was successively polished with 5, 1, 0.3 and 0.05 µm alumina polish (Buehler Ltd, USA) on a microcloth polishing cloth (Buehler) and then rinsed with 8 M nitric acid (Aldrich) and distilled water before use. All experiments were performed in 0.1 M acetate buffer adjusted to pH 5 with sodium hydroxide (Sigma) and all solutions were prepared with distilled water. Prior to analysis all solutions were purged with nitrogen for 15 minutes. All potentials were recorded versus the Ag|AgCl (3M NaCl) reference electrode and corrected for the normal hydrogen electrode (NHE).

## RESULTS AND DISCUSSION

For all the tested dyes and strains were obtained typical growth and decolourisation curves (31,33) with an acidification of the medium along the decolourisation process (results not shown). The fastest decrease in dye concentration in the medium was always observed in the late exponential growth phase as it was stated in an earlier publication by our group (31). The presence of dye in the medium did not affect the specific growth rates of the strains tested as it can be seen in figure 2A. There is also no toxic effect of these dyes and resulting degradation products since the re-inoculation of cells grown in the presence of dye results in a normal grow (results not show). Although specific growth rates are not significantly different (Figure 1A), the rate of dye degraded per gram of cell dry weight formed is considerably different (Figure 2B) as well as

the decolourisation times (Table 2). In Figure 2B and Table 2 it is also shown that the strain of *S. cerevisiae* tested is much more efficient in terms of decolourisation capability than the other two strains. The differences may be related to the mutation in the *CYR1* gene encoding the enzyme adenylate cyclase, but its relation with the azo reductase activity needs to be further explored (32).



**Figure 2.** (A) Specific growth rates and (B) rate of dye degraded for biomass formed of the strains *S. cerevisiae* CEN.PK113-7D, *I. occidentalis* PYCC5770 and *C. zeylanoides* UM2 in the presence of dyes I, II, III, IV and Amaranth grown in NDM with 0.2mM of dye at 26°C and 120 rpm.

**Table 2.** Decolourisation times in hours (for decolourisation  $\geq 98\%$ ) for strains *C. zeylanoides* UM2, *I. occidentalis* PYCC5770 and *S. cerevisiae* CEN.PK 113-7D for all the tested dyes.

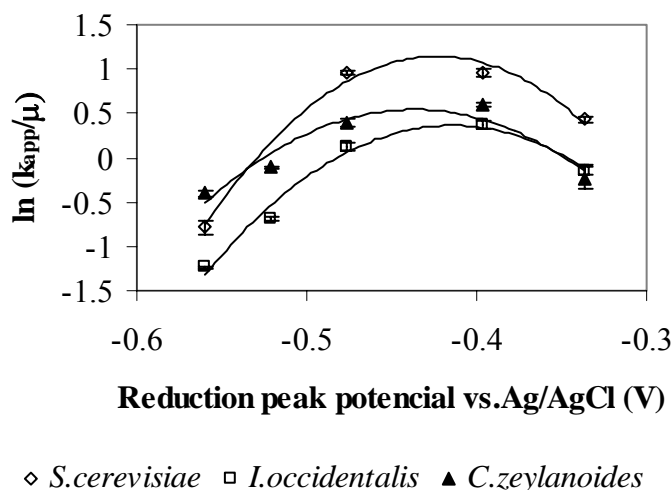
Dye	<i>C. zeylanoides</i> UM2	<i>I. occidentalis</i> PYCC5770	<i>S. cerevisiae</i> CEN.PK 113-7D
I	11	15	8.5
II	25	14	12
III	48	26	48
IV	24	30	78
Amaranth	12	15	8.5

To find an explanation for the differences observed in decolourisation abilities towards different dyes, the reduction potentials vs. Ag/AgCl of all tested dyes were determined. The results are depicted in Table 3.

**Table 3.** Reduction potential vs Ag/AgCl of the tested dyes.

Dye	Reduction potential vs. Ag/AgCl (V)
I	-0,397
II	-0,337
III	-0,521
IV	-0,560
Amaranth	-0,476

The primary data revealed a period of the growth phase in which decolourisation followed a 1<sup>st</sup> order equation. Since this constant is dependent on biomass growth, we have used a “normalized” parameter,  $k_{app}/\mu$ , where  $k_{app}$  stands for the apparent 1<sup>st</sup> order decolourisation rate constant and  $\mu$  for specific growth rate. Representing  $\ln(k_{app}/\mu)$  vs. the dye reduction peak potential it was obtained for the three strains a similar behaviour as it is shown in figure 3. This variation is represented by a second order polynomial equation. Dubin and Wright (16) and Bragger *et al.* (5) in studies with colonic bacteria found a linear relationship between the logarithm of the reduction rate and the redox potential of the tested dyes: the more positive the redox potential the more readily it is reduced. These observations are expected since the redox potential is a measure of the ease at which a molecule will accept electrons and be reduced. Semdé *et al.* (39) in studies with *Clostridium perfringens* found out that the degradation rate was independent of both the redox potential and the structure of the azo dye unless the dyes where introduced simultaneously. In this case the reduction would occur sequentially as a function of the substrate redox potential. However our results point to an energy-dependent decolourisation kinetics, although other factors as the charge of the dye molecule and the size of substituents may also affect the overall process. These results are not necessarily in disagreement with our previous findings (46) since we are studying different parameters.



**Figure 3.** Effect of the reduction peak potential of the tested dyes on the  $\ln$  (rate constant of apparent 1<sup>st</sup> order decolourisation ( $k_{app}$ ) / specific growth rate ( $\mu$ )). The results fit into the equations  $\ln(k_{app}/\mu) = -101.99E_{pc}^2 - 86.34E_{pc} - 17.13$  ( $r^2 = 0.9878$ ),  $\ln(k_{app}/\mu) = -79.39E_{pc}^2 - 65.82E_{pc} - 13.28$  ( $r^2 = 0.9777$ ),  $\ln(k_{app}/\mu) = -69.59E_{pc}^2 - 60.79E_{pc} - 12.73$  ( $r^2 = 0.8998$ ) respectively for *S. cerevisiae* CEN.PK 113-7D, *I. occidentalis* PYCC5770 and *C. zeylanoides* UM2 (where  $r^2$  stands for standard deviation error and  $E_{pc}$  stands for reduction peak potential of the dye).

The fate of the resulting amines was studied by assimilation experiments of these compounds in minimal media. The results are depicted in Table 4. In some cases the assimilation was preceded by a long lag phase (results not shown). This could represent the adaptation of the strain to the source. In terms of industrial

application of this process it is very important and significant that the yeast strains are able to use the resulting amines as carbon and nitrogen sources. These compounds are known to be carcinogenic and/or mutagenic (9). Therefore this ability of yeasts would allow to detoxify completely an wastewater contaminated with azo dyes. It is not described in literature one sole organism that is capable of both reducing the azo bond and mineralize the resulting amines under the same operational conditions. From the existing reports it can be seen that there are several limitations to the application of bacteria for the biodegradation of the aromatic amines formed from the reductive cleavage of the azo bond:

- the need for specific adapted strains (4,34) and in some cases co-cultures of several strains (12,20,43);
- chemical oxidation of some metabolites into more recalcitrant products (13);
- the lack of knowledge on the bio-reactivity of these compounds (30).

**Table 4.** Assimilation of the amines sulfanilic acid (SA), metanilic acid (MA), 1-amino-2-naphtol (1A2N) and N,N-dimethyl-p-phenylene diamine (NNDPFD) as sole source of carbon and energy (C) or nitrogen (N) by the yeast strains *Candida zeylanoides* UM2, *Issatchenkia occidentalis* PYCC5770 and *Saccharomyces cerevisiae* CEN.PK 113-7D.

Strain	SA		MA		1A2N		NNDPFD	
	C	N	C	N	C	N	C	N
<i>C. zeylanoides</i> UM2	+	+	+	+	+	+	+	+
<i>I. occidentalis</i> PYCC5770	+	+	+	+	+	+	+	+
<i>S. cerevisiae</i> CEN.PK 113-7D	+	+	+	+	+	+	+	+

(+) growth

In terms of industrial application is also important to test alternative and economic carbon sources. To achieve this and to further understand the process it was tested ethanol, starch, acetate, lactate, pyruvate and glycerol as carbon and energy sources in terms of growth and decolourisation effect. The results obtained are depicted in table 5.

**Table 5.** Effect of ethanol, starch, acetate, lactate, pyruvate and glycerol as carbon and energy sources in growth and decolourisation of dye I by the strains *S. cerevisiae* CEN.PK113-7D, *I. occidentalis* PYCC5770 and *C. zeylanoides* UM2.

C source	<i>S. cerevisiae</i> CEN.PK 113-7D		<i>I. occidentalis</i> PYCC5770		<i>C. zeylanoides</i> UM2	
	Growth	Decolourisation	Growth	Decolourisation	Growth	Decolourisation
Glucose	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+
Starch	-	-	-	-	-	-
Acetate	+	-	+	-	+	-
Lactate	+	-	+	-	+	-
Pyruvate	+	-	+	-	+	-
Glycerol	+	-	+	-	+	-

As it can be seen, only glucose and ethanol allowed both growth and decolourisation. The explanation for these observations can be that both glucose and ethanol metabolism have a higher yield in NADH (19), which is probably the intracellular electron donor for the dye reduction. We assume that the reduction of the azo

dyes by yeast cells is achieved by a reductase placed in the outer side of the plasma membrane of cells (32). This reductase transfers electrons between the intracellular donor (NAD(P)H) and the azo bound. It is so expected that the concentration/availability of the electron donor affects the reduction rate.

## CONCLUSIONS

This work provides evidence that yeasts are good bioremediation agents for azo dyes. Their growth and viability is not affected by the presence of dyes and their reduction products, which are potentially carcinogenic and mutagenic. Also they are able to use the produced amines as carbon and nitrogen sources, being able to achieve the desired complete mineralization of the azo compounds. The strain of *Saccharomyces cerevisiae* tested proved to be the most efficient decolouriser towards dyes I, II and Amaranth. When comparing the  $\ln(k_{app}/\mu)$  vs. the dye reduction peak potential, it was found that for all three strains that this relation was represented by a second order polynomial equation, suggesting that the decolourisation process is significantly affected by the reaction driving force.

## ACKNOWLEDGMENTS

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## **CHAPTER 7**

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### **DECOLOURISATION OF REACTIVE AZO DYES BY A BACTERIAL CONSORTIUM**

# Decolourisation of reactive azo dyes in alkaline textile effluents with a bacterial consortium

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## Abstract

The main goal of this work was to develop a continuous process to degrade azo dyes with a bacterial consortium isolated from a textile wastewater operating at pH 9 and 55°C. To assess the viability of the application of this treatment system to an industrial system several parameters were studied and related with the colour and COD removal efficiencies. The effects of hydraulic retention time (HRT), pH, temperature and presence of salts were studied. For the optimal operational conditions (pH 9 and HRT of 24 h) the efficiencies achieved were  $91.5 \pm 1.3\%$  for colour removal and  $89.0 \pm 0.4\%$  for COD removal. The system tolerated, with no significant decrease in colour removal efficiency 3% of  $\text{Na}_2\text{SO}_4$ ,  $\text{Na}_2\text{CO}_3$  or  $\text{NaCl}$ . The later salts, however, produced a reduction in COD removal of 30 and 50% respectively. The total suspended solids content in the outlet of the reactor changed widely during the operation. The mean value was  $0.54 \pm 0.22$  g/L. The system proved to be very effective in the decolourisation of C.I. Reactive Black 5 (RB5) under alkaline conditions and high temperatures.

**Short title:** Bacterial decolourisation of reactive azo dye

## 1. Introduction

Textile processing employs a variety of chemicals, depending on the nature of the raw material and products. The wastewater generated by different production steps has high pH and temperature. It also contains high concentration of organic matter, non-biodegradable matter, toxic substances, detergents and soaps, oil and grease, sulphide and alkalinity (1, 2, 3, 4). In addition, the high salt conditions of reactive dyebaths result in high-salt wastewater, which further exacerbates both their treatment and disposal.

Portuguese legislation establishes the limits for discharge of effluents from textile industry as follows: pH between 5.5 and 9 (Sorensen scale),  $\text{BOD}_5 < 100 \text{ mgO}_2/\text{L}$ ,  $\text{COD} < 250 \text{ mgO}_2/\text{L}$ , colour not visible at a dilution of 1:40 (5). These limits prevail over general regulations (6) for the discharge of residual waters.

Azo compounds constitute the largest and most diverse group of dyes and pigments used in commercial applications (7). During the production and use of these compounds a large amount is released to the environment, mainly to wastewaters. For instance in current cotton dyeing processes with reactive dyes as much as 50% of the

dye are lost in the wastewater (8). Their stability and xenobiotic nature make them very persistent in conventional treatment systems (9) and their presence in natural water courses above the natural depuration limit causes serious environmental problems. The reduction of the light absorption and the production of amines in anaerobic conditions are two of the problems that arise from the uncontrolled discharge of these compounds in water courses (9,10,11).

The major methods used for colour removal involve physical and/or chemical processes like oxidation, adsorption with different materials or microorganisms, membrane technology or coagulation/flocculation (for reviews on the subject see 12, 13, 14). These methods are highly expensive to apply and generate a concentrated sludge that creates disposal problems. They can also be the cause of a secondary pollution problem due to excessive chemical use (8). The conventional biological treatment systems like activated sludge are not capable of degrading the dyes, being mainly removed by physical adsorption (13, 15), because under aerobic conditions azo dyes are not readily metabolised (12). Also in these conventional systems, there are several substances like nitrate and nitrite, usually present in high levels in municipal wastewater, that compete with azo dyes as electron acceptors (16). This is why in

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the last few years a lot of research has been done in the azo dye biodegradation area mainly with bacteria, either isolated or in consortia, (8, 17, 10, 9, 12) and with white-rot fungi (18, 19). With yeasts there are only few reports that mention degradation rather than adsorption (20, 21, 22).

To develop a biological treatment system for these wastewaters there are several considerations to be made. It is known that the operational parameters of a biological treatment system greatly influence its efficiency. Parameters like hydraulic retention time (HRT), level of aeration, temperature and pH must be optimized to produce the maximum rate of dye reduction (8). Also the capability of using nutrients like acetate present in the effluents could help to reduce the cost and increase the applicability of a system in industry. Before being able to apply a treatment system to an industrial wastewater all the referred parameters should be studied and optimized.

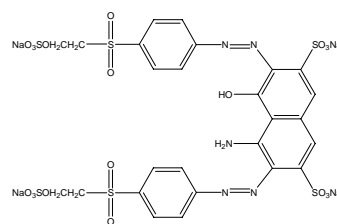
The main goal of this study was to assess the influence of several operational parameters on the colour and chemical oxygen demand (COD) removal efficiencies of a bacterial bioreactor to treat alkaline and coloured wastewaters at high temperature. The thermo-alkalophilic bacterial strain *Bacillus* sp. KF was selected based on the potential of its azoreductase to degrade azo dyes which we have reported earlier (23). The reactor was operated at pH 9 and 55°C because reactive dyes are used under alkaline conditions at high temperatures. The chosen dye was C.I. Reactive Black 5 (RB5), one of the most widely used in cotton dyeing processes. After some acclimation time it was found that there was a consortium present in the reactor, mainly constituted by *Bacillus* sp. KF, the initially inoculated strain. The use of consortia has several advantages for the use in an industrial treatment plant. Firstly there is no need for sterile conditions thus decreasing enormously the costs. These mixed population systems are also more stable to changes in pH, temperature and feeding composition when compared to pure cultures (13). Also there are more chances of a complete mineralization of the dye since in literature few strains are found that can alone metabolise completely this type of compounds (8).

## 2. Materials and methods

**Microorganisms.** The bacterial strain *Bacillus* sp. KF was isolated from a wastewater drain of a textile finishing company (Têxtil Alberto de Sousa, Guimarães, Portugal) based on its capability of degrading various dyestuffs at pH 9 and 60°C as

described by Maier et al. (23). After a period of acclimation it was found that there was a consortium of bacteria inside the reactor, mainly constituted by the *Bacillus* sp. strain (23).

**Chemicals.** Reactive Black 5 (RB5, min. dye content ~55%) was purchased from Aldrich and used without further purification (figure 1). Inorganic media components as well as glucose where from Merck. Peptone from casein and yeast extract where purchased from Difco.



**Figure 1:** Structure of CI Reactive Black 5.

**Medium for dye degradation.** Composition of the stock medium 10 times concentrated (concentrations in g/L)  $\text{KH}_2\text{PO}_4$  3,5;  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  7,5; yeast extract 10,0; peptone from casein 20,0;  $\text{NH}_4\text{SO}_4$  2,5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  4,5;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0,2 and iron citrate  $\cdot 5\text{H}_2\text{O}$  0,7; and 2,5 % (v/v) of a SL-6 trace element solution according to Pfennig (24). SL-6 contained (mg/L):  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  100,0;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  30,0;  $\text{H}_3\text{BO}_3$  300,0;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  10,0;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  20,0;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  30,0;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  200,0. To avoid precipitation of salts during autoclave sterilisation,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and iron citrate were autoclaved separately and aseptically combined with the other components. Medium pH was adjusted, except where stated otherwise, to  $9.0 \pm 0.2$  with NaOH. To prepare 1L of medium for the bioreactor, 100mL of this stock solution was diluted with 500mL of water and 400 mL of 1g/L RB5 solution.

**Bioreactor.** A scheme of the reactor used in this study is shown in figure 2. The vessel was 9 cm in diameter and 15 cm high (up to the conical top), and had an overall volume of 1.1 L. The volume of the filling support (Leca® Balls – air filled clay balls) was 0.65 L. The operation conditions, except where stated otherwise, were: a flux of 37.5 mL/h (corresponding to an HRT of 12 h), an aeration flux of 300 mL/min, and a temperature of c.a. 55°C. The system also included an aeration pump Tagus 3000, a feed pump Gilson model Minipuls2 and a heating bath at 60°C.

**Analytical methods.** The dye content in samples was calculated by absorbance measurements at 560nm against water. Samples were filtered with 0.20µm membranes and diluted as required. Total

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**Figure 2:** Scheme of the lab scale reactor for continuous decolourisation.

suspended solids (TSS), total solids (TS) and total volatile solids (TVS) were determined by standard gravimetric methods. Chemical oxygen demand (COD) was measured using a HACH spectrophotometer model DR/2000, after incubation on a HACH reactor, following the manufacturer's instructions for the HACH high range COD test (0 to 1500 ppm). Samples from the outlet were filtered with 0.20 $\mu$ m membranes prior to analysis to quantify only the dissolved COD.

### 3. Results and discussion

*Operation of the reactor.* The reactor was operated for 380 days. Each time the operation conditions were changed, the reactor was allowed to stabilize for four cycles (4 times the HRT) before the collection of samples. Samples were collected daily in the outlet of the reactor.

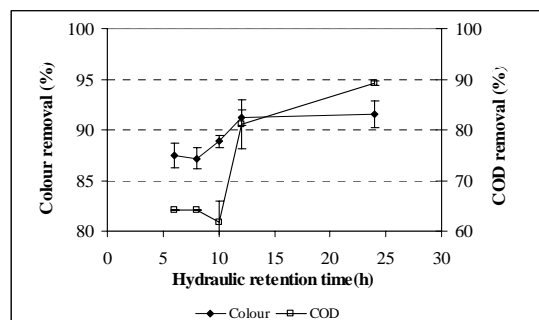
The total suspended solids content in the outlet of the reactor changed widely during the operation. The mean value was  $0.54 \pm 0.22$  g/L. This could be due to oscillations in the aeration flux because of frequent clogging problems and where mainly biomass leaving the reactor. For the application of this system in industry it would be necessary to apply a sedimentation process in the outlet of the reactor since the amount of solids is very high (for discharge in aquatic environment, in Portugal, the maximum amount allowed is 60mg/L, 6). This amount of solids in the outlet is not totally unexpected since the degradation achieved is high, meaning that the production of biomass is also high.

The biomass present inside the reactor as volatile solids (VS) was determined and a mean value of  $16 \pm 3.3$ gVS was achieved. The support carried an average of  $20.6 \pm 4.3$  mgVS/g support.

For this work the support chosen was Leca® balls due to its low cost, widespread availability, high porosity, mechanical resistance and resistance to high pH.

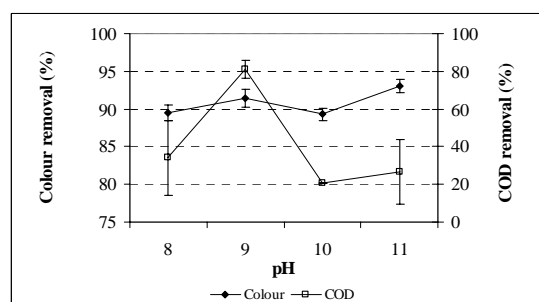
*Effect of the hydraulic residence time (HRT).* To study the effect of the HRT on the reactor performance this parameter was changed between 6 and 24h. The results on the percentage of colour removal are depicted in figure 3. Between 6 and 12h there is a significant increase in the colour removal efficiency, but above 12h there is no further increase. The efficiency of the COD removal is displayed against the HRT in the same figure. COD removal remained almost constant at

HRT of 6-10h but increased almost 20% at 12h HRT. The low COD removal for low HRT is expected due to the incomplete consumption of the nutrients in the medium which will account for the total COD amount.



**Figure 3:** Effect of the hydraulic retention time on the colour and COD removal efficiencies for the operation of the reactor at pH 9 and 55°C.

*Effect of the pH.* As the isolated bacteria were alkalophilic the effect of pH was studied between 8 and 11 for a hydraulic retention time of 12 h. The results are depicted in figure 4. Within the tested range dye removal did not significantly changed, but for COD removal a maximum was observed at pH 9.

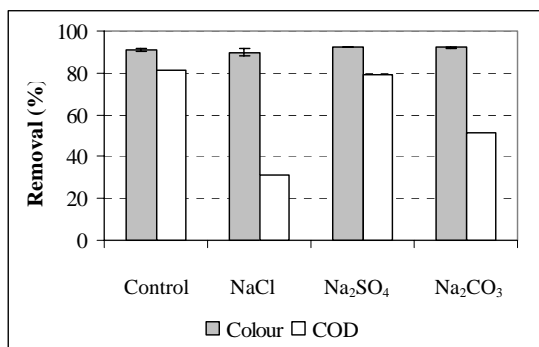


**Figure 4:** Effect of pH on the colour and COD removal efficiencies for the operation of the reactor at 12 h of hydraulic retention time and 55°C.

*Effect of temperature.* The effect of temperature was only tested at two values: room temperature, 20°C, and at 55°C. The differences observed were not significant. At 20°C the percentage of colour removal was  $88.2 \pm 1.2$  whereas for 55°C it was  $87.9 \pm 2.0$ . The effect on COD removal was not determined. In many systems, the rate of colour removal increases with increasing temperature, within a defined range that depends on the system (8). The temperature required to produce the maximum rate of colour removal tends to correlate to the optimum cell culture growth temperature. The decline in colour removal activity at higher temperatures can be attributed to the loss of cell viability or to the denaturation of the azo reductase enzyme(s). However the immobilisation of the cell

culture in a support medium usually results in a shift in the optimum colour removal temperature towards high values because the microenvironment inside the support offers protection for the cells. This could be, along with the thermophilic character of the strain, the reason for the small difference observed in the removal efficiency.

**Effect of the presence of salts.** The salts tested were NaCl, Na<sub>2</sub>SO<sub>4</sub> and Na<sub>2</sub>CO<sub>3</sub> at 3% (w/v). These salts are used mainly in dyeing processes as auxiliaries to ensure maximum fixation of dye to the fiber (3,25). The results are depicted in figure 5. From the results obtained for colour removal we can say that apparently there is no effect of salts on colour removal. On the other hand, the effect on COD removal is negative particularly for NaCl. Isik (25) observed the same type of behaviour in an upflow anaerobic sludge blanket reactor. High salt concentrations (>1% salt) are known to cause plasmolysis and/or loss of cell activity (26). This would explain the decrease in COD removal efficiency. However since efficiency in colour removal is not significantly decreased it is possible that under these conditions the cells are not growing but still capable of working as biocatalysts. This means that this bacterial consortium presents a high tolerance to salt



**Figure 5:** Effect of the presence of 3% of NaCl, Na<sub>2</sub>SO<sub>4</sub> or Na<sub>2</sub>CO<sub>3</sub> on colour and COD removal efficiencies for the operation of the reactor at 12 h of hydraulic retention time, pH 9 and 55°C.

concentration, which represents an advantage of this system for the treatment of high-salt concentration wastewaters.

#### 4. Conclusions

The results obtained in this study allowed us to establish the optimal operational conditions for a continuous reactor degrading textile dyes based on a consortium of alkalothermophilic *Bacillus* sp. being: pH 9 and HRT of 24 h. For these conditions the efficiencies achieved were 91.5±1.3% of colour

removal and 89.0±0.4% of COD removal. This bacterial system proved to be salt tolerant being affected to a bigger extent by NaCl. Also it was shown that oscillations in temperature between 20 and 55°C did not affect its performance in terms of colour removal. The fate of the produced amines was not explored since the objective was mainly the reduction of colour and there are conventional aerobic systems that are able to fully mineralize these types of compounds. TSS in the outlet varied considerably in the outlet, and the mean value obtained during the whole reactor operation was 0.54±0.22 g/L.

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## **CHAPTER 8**

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### **GENERAL DISCUSSION & FUTURE PRESPECTIVES**



## GENERAL DISCUSSION

Over the last years because of stringent legislation aiming the protection of the environment, several systems for azo dye removal have been developed and explored. Among the biological methods, the ones using bacteria and white-rot fungi are the most widely studied. The use of yeasts to achieve this objective has never been subject of investigation, probably because the few studies on the subject mention biosorption as removal mechanism. When we isolated from contaminated soil several ascomycete strains we decided to explore their potential on azo dye bioremediation. We started with ascomycete yeasts *Candida zeylanoides* and *Issatchenkia occidentalis*. Later, to study the enzymatic system involved, a laboratorial strain was selected, *Saccharomyces cerevisiae* CEN.PK 113-7D. In most studies the dyes *m*-[(4-dimethylamino)phenylazo]benzenesulfonic acid, *p*-[(4-dimethylamino)phenylazo]benzenesulfonic acid (Methyl Orange), *m*-[(2-hydroxy-1-naphthyl)azo]benzenesulfonic acid, *p*-[(2-hydroxy-1-naphthyl)azo]benzenesulfonic acid (Orange II) and Amaranth were used.

The optimum growth and decolourisation conditions (incubation at 26°C and 120 rpm with 0.2mM of azo dye and 2% of glucose as carbon and energy source) were established for *C. zeylanoides* (chapter 2) and later used with *I. occidentalis* and *S. cerevisiae*. None of the tested azo dyes supported growth as sole source of carbon and nitrogen (chapters 2 and 6). This means that external C and N sources must be present in the medium to achieve decolourisation and that azo dyes are subject to cometabolism (fortuitous metabolism) by yeasts (Knackmuss 1996).

The presence of dyes did not significantly affect yeast growth and exposure of the cells to the dye reduction products (aromatic amines) did not decrease their viability (chapters 2, 3 and 6). This fact is important for the applicability of the system to the treatment of an industrial wastewater.

Colour removal was achieved between 11 and 48h of incubation by *C. zeylanoides*, between 14 and 30h by *I. occidentalis* and between 8.5 and 78h by *S. cerevisiae* (chapter 6). The decolourisation progress was affected by dye structure including the different position of identical substituents (chapter 2, 3 and 6). These are fast removal rates considering for instance the 15 days reported for *P. chrysosporium* by Pasti-Grigsby and co-workers for Orange II (Pasti-Grigsby *et al.* 1992).

The mechanism of colour removal was found to be the reductive cleavage of the azo bound with formation of aromatic amines (chapters 2, 3 and 5). The first study with *C. zeylanoides* carried out with the resulting amines showed that the two aminobenzenesulfonic acids (metanilic and sulfanilic acids) were used as nitrogen but not as carbon and energy sources (chapter 2). Subsequent studies with *I. occidentalis* showed that these two aminobenzene

sulfonic acids were not used but N,N-dimethyl-*p*-phenylene diamine was used as nitrogen source and that 1-amino-2-naphtol was used as nitrogen and carbon and energy (chapter 3). Later studies, with longer exposure times, proved that all the formed amines (metanilic and sulfanilic acids, 1-amino-2-naphtol and N,N-dimethyl-*p*-phenylene diamine) could be used as carbon and nitrogen sources (chapter 6). These results are very important in terms of industrial application of this system because it achieves the complete mineralization of the dyes under the same operational conditions. There is no single microorganism described with these characteristics. To achieve the complete mineralization of the formed amines probably hydraulic retention times longer than the ones to achieve decolourisation will be required.

All the tested strains fermented glucose, as confirmed by detection of ethanol in the supernatants (chapter 2 and 3). When using methyl orange the decolourisation capability was not exhausted after one decolourisation cycle (chapter 3). In fact, dye was decolourised completely over 5 dye addition cycles and the 6<sup>th</sup> addition was not completely decolourised because after *c.a.* 160h ethanol was exhausted from the medium. This experiment demonstrates that ethanol can be used as carbon and energy source for dye decolourisation.

Several other carbon sources were tested (like acetate and starch) to reduce the cost of nutrients in the system, but only ethanol allowed both growth and decolourisation (chapter 3 and 6).

The decolourising ability was not affected by previous exposure of the cells to dye, strongly suggesting that it is a constitutive activity (chapter 2 and 3). There was no detectable reductase activity in the supernatants or in cell extracts (chapter 3). Also the decolourisation in the presence of glucose was always connected with the late exponential growth phase (chapters 2, 3 and 6). The suspicion that we were dealing with an enzymatic system was confirmed by the results and by the knowledge that polar dyes are impermeant to cell membranes and their transformation by living microbial cells must occur in the extracellular medium (Pearce *et al.* 2003). An enzymatic assay was developed in order to study the “azoreductase” activity (chapter 3). The specific decolourisation rate obeyed to the Michaelis-Menten equation with a maximum specific decolourisation rate of  $3.2\text{mmol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$  and a  $K_m$  of 0.034mM for methyl orange (chapter 3). The bell-shaped form, characteristic of an enzymatic system, was found to describe the dependency of the decolourisation rate on temperature. The maximum activity was obtained at 50°C, but for 60°C it was observed a complete loss of activity (chapter 3). pH affected differently the decolourisation rates of N,N-dimethylaniline-based dyes and  $\beta$ -naphthol-based dyes: the first were practically independent on pH between 3 and 6, showing a rapid decrease between 6 and 7; the second showed a linear decrease between 3 and 6, being undetectable at the latter value (chapter 3).

Dissolved oxygen is repeatedly considered an inhibitor of the azo dye reduction process (Pearce *et al.* 2003), since both molecules act as electron acceptors and oxygen is a much stronger

oxidant. This is apparently the reason why azo dyes are more readily reduced under anaerobic conditions. We decided to check the oxygen concentration in the medium during decolourisation (chapter 3). In our conditions, i.e., cotton plugged 250 mL flasks with 100 mL of medium shaken at 120 rpm, oxygen accesses the medium. Nevertheless, after only 6h of growth the dissolved oxygen concentration is kept under the detection level ( $0.2\text{mg.L}^{-1}$ ) until the end of the experiment. Therefore decolourisation occurs under very low oxygen concentrations. To explore this aspect assays were performed under: our standard conditions – 120 rpm (microaerophilic), forced aeration (aerobic) and  $\text{N}_2$  flushed (anaerobic). It was concluded that:

- oxygen is needed for the respiration of ethanol when glucose is extinguished from the growth medium although in very small concentrations (under anaerobic conditions yeasts failed to grow);
- under forced aeration conditions and under microaerophilic conditions the decolourisation progress is similar showing that in this system oxygen does not compete with the dye for electrons.

These results are important since if forced aeration is not needed the industrial implementation of this system is easier (energy costs due to forced aeration are usually very high).

In an attempt to rationalize the different decolourisation rates of the tested dyes, further electrochemical studies showed a linear relation between the time to achieve decolourisation above 98% and the cathodic peak potential (*vs.* SHE) of the azo dye. The less negative the reduction potential of the azo dye, the more favourable will be its reduction (chapter 4). Our last studies found that the relationship of  $\ln(\text{apparent first order decolourisation rate/specific growth rate})$  *vs.* the reduction peak potential was represented by a second order polynomial equation for the three strains and the five dyes tested. This type of relation suggests that the decolourisation kinetics is energy-dependent (chapter 6).

Since we were searching for a plasma membrane redox system and in yeast the more studied is the ferric reductase system it was decided to investigate if this activity could be connected to the “azoreductase” activity (chapter 5). The results showed that these two activities are the same and are achieved by the Fre1p of the plasma membrane in our operational conditions. The results that pointed out to this conclusion were:

- both activities had closely parallel curves along growth although with significant differences in absolute values;
- iron inhibited both activities;
- the deletion of *FRE1* abolished almost completely both activities;
- the azoreductase activity was recovered when the gene was reintroduced in the yeast.

This knowledge allow us to control better the azoreductase activity and in a near future to improve strains for better performances. Also gives a contribution for the elucidation of

multifunctional plasma membrane redox systems in yeasts that are scarcely explored and are involved in almost all functions in all types of cells.

We can point the major advantages of the use of these microorganisms for the bioremediation of azo dyes:

- low cost of the system: aeration is not necessary;
- non patogenicity of the microorganisms used;
- the capability of degradation and mineralization of the dyes under the same conditions;
- the know genome opens possibilities of genetic manipulations in order to increase the system performance;
- low sludge generation (low growth rates) with no disposal problem;

Some of the major disadvantages are:

- low growth rates of yeasts that makes the system vulnerable to contaminations by microorganisms with higher growth rates, like bacteria;
- the incapacity of degrading dyes above pH 5 (usually the pH of this wastewaters is very alkaline);

This system is efficient for the decolourisation of acid azo dye containing wastewaters although further studies are needed to access the effect of some parameters in a continuous system (like salts and other components typical in these wastewaters).

In the last part (chapter 7) of this thesis there is a study of a thermophilic bacterial consortium in a continuous reactor to decolourize a simulated wastewater with Reactive Black 5. The bacteria *Bacillus* sp. KF was isolated from contaminated wastewater and the reactor was designed in Austria by the group of Prof. Georg Gübitz. The idea was to build a similar reactor with yeasts and compare the efficiencies of both systems, one alkalophilic and the other acidophilic. Unfortunately due to lack of time the yeast reactor was not build. Nevertheless the operation with bacterial consortium allowed several conclusions:

- a high degradation rate is achieved (92% of colour removal and 89% of COD removal for the optimum operation conditions, i.e., pH 9 and HRT of 24h) with the concomitant high sludge production;
- the system tolerates high salts concentrations (up to 3% of NaCl, Na<sub>2</sub>SO<sub>4</sub> and Na<sub>2</sub>CO<sub>3</sub>) with no significant reduction in colour removal efficiency, but with the same negative effect in COD removal, especially in the presence of NaCl;
- the system tolerated with no significant decrease in the colour removal efficiency temperatures between 20 and 55°C, probably due to the thermophilic character of

the *Bacillus* sp. KF strain and to the protective environment inside the support material;

This system can efficiently degrade Reactive Black 5 dye under alkalophilic conditions.

## FUTURE PRESPECTIVES

We believe that this work provides a starting point for more intensive study to better understand the broader non-specific activities associated with this plasma membrane redox mechanism, as well as future clues for the biotechnological optimization and exploitation of the activity.

We have established that the “azoreductase” activity of *Saccharomyces cerevisiae* cells is dependent on Fre1p. However ferric reductase activity is a complex system requiring other components and subject to fine regulation. Therefore it would be particularly interesting to investigate further aspects of the system:

- 1) the effect of known inhibitors of ferric reductase on the azoreductase activity;
- 2) the nature of the electron donor;
- 3) participation of other components in the *in vivo* “azoreductase” activity;
- 4) effect of the ras/cAMP pathway on the regulation of the “azoreductase” activity;
- 5) since there are other redox systems in the plasma membrane of *S. cerevisiae* it would be interesting to investigate their possible participation in the “azoreductase” activity in different conditions from those studied in this work.

This would be important not only in the use of yeasts as bioremediation agents but also in human health and drug development.

Additionally the biodegradation of azo dyes is a process relevant to the treatment of wastewaters from textile industries which are frequently heavily coloured and resistant to conventional wastewater treatment processes. The design and operation of a continuous reactor with yeasts to study the effect of several parameters in its operation, namely pH, temperature and presence of salts and other textile additives would undoubtedly reveal more aspects of this system. This would allow the development of a pilot treatment plant to apply in a textile industry.

Another possible development of this work would be to go on exploring the capability of other yeasts as bioremediation agents of textile dyes, particularly the basidiomycetes isolated from contaminated soil in the beginning of these studies. Also those with oxidative capabilities can be explored and be eventually useful for the degradation of other types of dyes. We can also explore some thermophilic strains for the treatment at higher temperatures and strains with a wider operational pH.

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